BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12Q 1/68, 1/00, G01N 33/50
G01N 33/48, 33/49, C07H 19/067

A1

(11) International Publication Number: WO 86/ 05815

(43) International Publicati n Date: 9 October 1986 (09.10.86)

(21) International Application Number: PCT/GB86/00174

(22) International Filing Date: 25 March 1986 (25.03.86)

(31) Priority Application Number: 8507706

(32) Priority Date: 25 March 1985 (25.03.85)

(33) Priority Country: GB

(71) Applicant *(for all designated States except US)*: GENETICS INTERNATIONAL INC. [US/GB]; 11 Nuffield Way, Abingdon, Oxon OX14 1RL (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : HILL, Hugh, Alan, Oliver [GB/GB]; 9 Glover Close, Cunmor, Oxfordshire OX2 9JH (GB). GEAR, Mark, Jonathan [GB/GB]; Room 301A, 25 Wellington Square, Oxford OX1 2JH (GB). WILLIAMS, Stephen, Charles [GB/GB]; 3 Acacia Gardens, Southmoor, Oxon (GB). GREEN, Monika, Joanna [GB/GB]; 2 Long Row, Beckhamstead, Buckinghamshire (GB).

(74) Agent: CLIFFORD, Frederick, Alan; Marks & Clerk, 57/60 Lincolns Inn Fields, London WC2A 3LS (GB).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NUCLEIC ACID SEQUENCES ATTACHED TO MATERIALS SENSITIVE TO MAGNETIC FIELDS, AND METHODS OF ASSAY AND APPARATUS USING SUCH ATTACHED SEQUENCES.

(57) Abstract

Magnetic particles coated with cellulose derivatives, e.g. nitrocellulose, link as a support to DNA or RNA sequences. A probe sequence, itself labelled, e.g. with biotin, hybridizes with suitable complementary supported sequences, and the resulting complexes are magnetically separated. Avidin, or streptavidin, then couples with the biotin and the complex is again magnetically separated. The avidin can be already linked with a marker enzyme such as horseradish peroxide, or glucose oxidase, or alkaline phosphatase, or can be subsequently linked thereto. In either case, subsequent contact with a suitable substrate for the enzyme (H_2O_2, glucose, phenylphosphate) gives a reaction for electrochemical measurement either via a ferrocene mediator compound (H_2O_2, glucose) or by oxidation at an electrode surface (phenol from phenyl phosphate). This reaction eventually relates back very sensitively to the presence or amount of initial hybridisation; attomole quantities are measurable.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GA**	Gabon	**MR**	Mauritania
AU	Australia	**GB**	United Kingdom	**MW**	Malawi
BB	Barbados	**HU**	Hungary	**NL**	Netherlands
BE	Belgium	**IT**	Italy	**NO**	Norway
BG	Bulgaria	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		
FR	France	**ML**	Mali		

Title: NUCLEIC ACID SEQUENCES ATTACHED TO MATERIALS SENSITIVE TO MAGNETIC FIELDS, AND METHODS OF ASSAY AND APPARATUS USING SUCH ATTACHED SEQUENCES.

The present invention is concerned with nucleic acid sequences, assays for nucleic acids and apparatus for such assay.

The assay procedures of the present invention extend to both to detection of the presence of nucleic acids and to the identification of specific nucleic acid molecules, or classes of molecules, by identifying sequences therein, both procedures being needed in many areas of biochemical research and commerce.

Hybridisation between two single-stranded DNA (or RNA) molecules, which have complementary (or partially complementary) sequences has been one approach to the problem of nucleic acid assay. Molecules for use in such assays are capable of binding to a DNA (or RNA) sequence defined to a particular level of sequence homology, and are known as "DNA probes"(or "RNA probes")

Known DNA (RNA) probe techniques all have the

characteristic that the DNA (RNA) polymer formed by hydridisation between the probe and target sequence is not readily detectable by its inherent biochemical activity. It is therefore necessary to mark the polymer formed with some signal-producing chemical or biochemical species.

One particular known method of marking is commonly known as the "Avidin-Biotin Reaction" and relies on the affinity of the egg-white glycoprotein avidin for biotin.

In this method biotin (Vitamin H) is chemically linked to one or more nucleotides, i.e residues which comprise the monomeric units of a DNA polymer. (For convenience of description reference below to DNA probes should be taken to refer also to RNA probes). A single-stranded sequence of a DNA is built up using known units in a known sequence, at least one such being biotin-linked. The sequence still undergoes the classical hybridisation reaction with a complementary strand of DNA to form a double-stranded DNA. It can thus be incorporated into synthetic DNA probes.

In the majority of DNA probe assays, any remaining unbound probe must be separated from the bound probe . This separation is normally done by performing the

hybridisation reaction in conditions under which sample DNA including the target sequence is immobilized on a substrate and following the binding reaction by washing, or centrifugation to remove any excess probe. The bound probe is therefore all that remains, as an indication or measure of the target sequence. It is detected by the addition of avidin, or streptavidin, to which a marker has been attached.

Typically, the marker is an enzyme and is for example horse radish peroxidase or alkaline phosphatase. The marker enzymeis then "developed" by reaction with a substrate which produces a coloured, insoluble precipitate on the membrane surface; the extent of the marker enzyme reaction is usually estimated by eye, or more rarely by densitometry, and although this method provides sensitivity down to the picogram level (Leary et al Proc. Natl. Acad. Sci. USA 80 4045-4049,1983, Chan et al, Nucleic Acids Research 10 8083-8091) it is inherently insensitive and fails to distinguish between single and double copy gene levels.

Our present invention, however, involves a variant on such method of marking the probe sequence, which can also utilise the technology described in our copending European Patent Application 82305597, relating to

methods of assay in which a mediator compound is used to transfer charge from an enzyme to an electrode. Such a system as therein described may be employed to detect the concentration of either a substrate or an enzyme in a sample, by electrochemical measurement of the occurrence or extent of an enzyme-affected reaction. Moreover,our copending European Patent Application 84.303090 discloses a novel route to the measurement of nucleic acid probe sequence coupling by using a method developed from the above in which at least one of a mediator and an enzyme, for use in such electrochemical assay are chemically linked to a nucleic acid probe sequence and where any specific binding, of the probe sequence, to the target sequence which may take place thus affects the electrochemical availability of the chemically linked species, for detection by a sensor electrode in presence of the enzyme substrate. Such technology is also relevant to a consideration of the present invention.

One aim of the DNA probe technology and the enzyme detection/assay technology so far developed, has been to detect "inbuilt errors of metabolism" which lead to a variety of "genetic diseases" and inheritable disorders. Among such disorders are: Familial Goiter (iodotyrosin dehalogenase defective), Maple syrup urine dis ase (alpha-keto decarboxylase defectiv),

Xanthinuria (Xanthine oxidase defective) and Methaemoglobinemia (Methaemoglobin reductase defective).

A full list of over 3500 conditions due to defective genes can be found in McKusick's "Mendelian Inheritance in Man".

Clearly, in the detection of simple mutations it is necessary to select and recognise relatively short and specific nucleic acid sequences from the entire genetic library of the organism in question. In general, therefore, and in the methods of the prior art mentioned above, a binding reaction must be followed by a recognition step in which the hybridisation event is detected and occasionally quantified.

It will be noted from the above description that there is a requirement for separation of various components of the assay mixture at some stage in the method. One requirement for example is the separation of unbound probe DNA from the probe/target conjugate. This separation requirement has tended to make heterogeneous assays either slow, cumbersome and expensive and has needed skilled technicians. Furthermore, although we have developed assays e.g.using the mediator/redox

systems described in general terms above, a separation and/or concentration step increases the resolution of these assays; there is still a preference if not a need for separation.

The present invention sets out to provide materials, techniques and apparatus to improve the ease of separation in such assays.

According to one aspect of the present invention there is provided an entity including a single or double-stranded nucleic acid sequence linked to a magnetic or magnetisable substance.

Usually, the invention is concerned with a single-strand sequence linked to a magnetic or magnetisable substance, the sequence being a DNA or RNA sequence in a pure or impure form, as obtainable from a natural or a synthetic source. Pure and known sequences will generally comprise a "probe" sequence, but of course a target sequence could be (and usually is) linked to the magnetic or magnetisable substance in accordance with the present invention. Moreover the hybridised or other double stranded DNA (RNA) magnetic/magnetisable entity can also constitute a feature of the invention.

The magnetic/magnetisable substance is usually provided

in a divided or particulate form.

Typically, in this aspect of the present invention, a magnetic or magnetisable particle is bound to the target nucleic acid sequence.

In a preferred form of the invention a substance having a permanent magnetic moment, i.e. a ferromagnetic material, especially iron particles or ferrite particles or magnetite particles, Fe_3O_4, is treated in such a way that the sequence will subsequently attach thereto.

In some embodiments, this attachment can be effected by simple adsorption or by chemical cross-linking directly to the magnetic material, but a more preferable product has a coating on the magnetic substance of a material to which a nucleic acid material can become permanently attached, for example nitrocellulose,and a nucleic acid attached to this coating .Nucleic acids attached to nitrocellulose are known in the chemical art.

The invention also extends to the use of particles of a magnetic or magnetisable substance, coated with a material capable of attachment to a DNA or RNA single-strand material, in a magnetic separation under the influence of an applied magnetic field for the

purpose of separating (a) such nucleic acids from a mixture of materials or (b) attached nucleic acids hybridised with a nucleic acid containing a complementary sequence from excess of the unattached complementary nucleic acid or (c) such attached and hybridised materials from an excess of an enzyme marker, or of a material convertible to an enzyme marker, reactive therewith.

In another aspect of the present invention there is provided a method of separation of single-stranded nucleic acid materials from a complex mixture containing such materials, in which the mixture is treated with magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and the particles and attached nucleic acid materials are thereafter separated from the other components, at least in part, by a magnetic field. As indicated above the coating may be nitrocellulose, but cellulose itself (which generally provides functional groups which can be readily derivatised allowing stable covalent attachment of nucleic acids) can be used.

In yet another aspect of the present invention there is provided a method for the detection or quantification of the pr sence of a target s quence of nucleic acid units,

or number of such sequences , in a single-stranded DNA or RNA material or mixture of such materials, by contact with a probe DNA or RNA sequence of a predetermined level of homology to the target sequences, followed by separation of the materials for detection or quantification of the probe/target hybridisation reaction; in which the probe sequence or the target sequence is a single-stranded nucleic acid sequence linked to a magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and in which separation is at least in part effected by a magnetic field.

The present invention is particularly concerned with a method for the detection or quantification of the presence of a target sequence of nucleic acid units, or number of said sequences, in a single-stranded RNA or DNA material, or mixture of such materials, in the presence of a complex liquid mixture containing inter alia such DNA or RNA single-stranded materials, comprising the steps of:

(i) contacting the complex liquid mixture with a magnetic or magnetisable material in the form of particles having a coating to which any nucleic acid single-strand RNA or DNA material becomes permanently attached, and thereafter separating the

magnetic particles from the mixture, at least in part, by a magnetic field,

(ii)contacting the separated DNA/RNA material linked to the magnetic or magnetisable particle with a probe single-stranded DNA or RNA sequence to detect or quantify by a hybridisation reaction the presence of the target sequence on said permanently attached DNA/RNA magnetically separated material, and thereafter separating the magnetic or magnetisable material from unbound probe sequence, at least in part, by a magnetic field,

prior to assay of the probe/target bound species.

The probe may be isolated from an organism, or be chemically synthesised, or be synthesised using a host organism.

Assay of the probe-target bound species can be effected by known methods. For example, the probe can be chemically linked with compound A of a pair of compounds A and B which themselves react with a specific binding reaction thus useful for measuring probe level in the separated magnetic materials. Examples of A and B are antigen/antibody; hormone/receptor; lectin/carbohydrate; or cofactor and enzyme all of which give a known and measurable type of assay for the prob content. A

preferred pair of materials A and B are however biotin and avidin, or possibly biotin and streptavidin; the reaction is well known in this context and measurement methods involving fluorescence or enzyme reactions (of an enzyme linked to the avidin) are well-established.

Nonetheless, we have further established a preferred final measurement method preferably using the biotin/avidin, or biotin/streptavidin specific binding system. The method involves attaching biotin to the probe and a suitable e.g. redox enzyme to the avidin or streptravidin.On addition of suitable substrate a product is formed . The extent or occurrence of this reaction can be measured at an electrode directly or conveyed to an electrode by a suitable mediator such as a metallocene, more especially a ferrocene as disclosed in our earlier Applications itemised above.

The enzyme can be glucose oxidase, or glucose dehydrogenase, for use with glucose as a substrate. The preferred enzyme is however a peroxidase e.g. horseradish peroxidase, for use with H_2O_2 as a substrate. More generally speaking, any redox enzyme can be measured, e.g. in addition to the above, dehydrogenases or those enzymes capable of attacking or degrading large molecules e.g. α-amylase, RNA-ases or DNA-ases.

It is believed however that many of the nzyme-substrate pairs disclosed in the Applications identified above which we have studied could be utilised in association with the mediator in this aspect of the method of the present invention, given some limitations on the assay conditions which would be obvious to the man skilled in the art. One example of such a necessary variation would be if the substrate of one particular enzyme were for some reason present in the assay mixture. Of these possible pairs, it is clearly advantageous to utilise those enzyme/substrate pairs whose behaviour is established in most detail and which give good, and preferably linear, response over the expected measurement range.

Ferrocenes (bis cyclopentadienyl iron and its derivatives) have chemical and electrochemical advantages over other mediators used with enzyme/ substrate reactions for charge-transfer purposes.A number of ferrocene compounds are disclosed in more detail below.

The enzyme of particular preference is glucose oxidase although the enzyme horse radish peroxidase may also be used. The mediator of preference is ferrocene or a derivative thereof. Suitable protocols for the measurement of glucose oxidase concentration by an

electrochemical method in the presence of ferrocene are given in the above referenced patent applications.

As described in the patent applications, identified above,it is possible to detect the presence of oxidoreductases using amperometry or coulometry, by coupling the electron transfer from the substrate to the enzyme and thence to a sensor electrode. Such an electrode is generally referred to as an "electron transfer electrode".

In a specific embodiment of the present invention therefore there is provided a method for detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a celluose derivatives (DCEL) such as nitrocellactose (NITC) whereby single-stranded nucleic acids (NUCA) become permanently attached thereto, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(ii) contacting the (NUCA)-(NITC)-(MAG) material thus separated with a single-stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the

(NUCA)-(DCEL)-(MAG) particles possessing a complementary target sequence will react, with hybridisation to give the complex (BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field

(iii) contacting the separated materials with avidin or streptavidin (AV) linked with a peroxidase enzyme (PER) to form the complex (PER)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field, and

(iv) bringing the said magnetically separated materials carrying the said complex into contact with hydrogen peroxide and a ferrocene, whereby the terminal (PER) group on the complex causes the hydrogen peroxide to react so that the ferrocene transfers charge to or from acontacting electrode to feed a readout signal.

In another variant of this method of measurement the preferred particle coating is cellulose, and the enzyme is an alkaline phosphatase, capable of producing phenol, for oxidation at a working electrode, from a phenyl phosphate substrate. Such an enzyme can be itself linked by biotin to the avidin (or streptavidin) after the biotin-avidin reaction on the hybridised material.

Thus, in one form of the invention there is provided a method for the detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a derivatised cellulose (DCEL) capable of permanently attaching thereto single-stranded nucleic acids, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(ii) contacting the (NUCA)-(DCEL)-(MAG) material thus separated with a single stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the (NUCA)-(DCEL)-(MAG) particles possessing a complementary target sequence will react with hybridisation to give the complex (BIO)-(PRO)-(NUCA)-(DCEL)-MAG, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iii) contacting the separated materials with avidin or streptavidin (AV) to form the complex (AV)-(BIO)-(PRO)-(NUCA)-DCEL)-(MAG) and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iv) thereafter linking to the (AV)-groups of the

complex a marker enzyme (ENZ), and

(v) bringing the resultant (ENC)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG into contact with a suitable substrate for the said enzyme and measuring the enzyme reaction electrochemically.

The cellulose derivative (DCEL) can be an aminophenylthioether linkage, activated to the corresponding diazo compound for immobilisation of the single-strand (NUCA).

The (AV) group can be itself - biotin-linked to the further enzyme, which in one preferred embodiment is an alkaline phosphatase utilising phenyl phosphate as a substrate convertible to phenol, or other substrates. In such a case direct, unmediated, measurement is possible.

The invention further extends to apparatus for use in the method of assay as defined above comprising an upper vessel, a lower vessel selectively placeable in liquid flow communication therewith, and selectively applicable magnetic element in said upper vessel. Possibly the magnetic element is a magnetisable, or magnetic, grid element having two layers relatively movable to provide either separation or communication between the vessels.

Alternatively, the magnetic element may be an electrode for subsequent use in the electrochemical determination of the enzyme reaction; if so it has the potential advantage of drawing towards itself the magnetic species.

According to the invention therefore, if separation procedures (including washing procedures) are required, use may be made of the magnetic properties of the various complexes either by placing tubes containing the complexes in a magnetic or magnetisable test-tube rack or by making use of a preferred apparatus disclosed below. In this manner substances which have not become linked to the coated magnetic particles may be removed from the assay sample. Thus the target DNA or RNA may be separated from debris such as other broken-cell products, and buffer or other solutions.

The invention will be further described by way of example and with reference to the accompanying drawings, wherein:

Figure 1 diagrammatically illustrates steps of an assay method according to the present invention,

Figures 2a and 2b show features of an apparatus according to the present invention,generally in vertical

cross-section.

Figure 3, in its three component parts, shows diagrammatically steps in a method of assay of the present invention carried out in apparatus according to the present invention,

Figure 4, in its three component parts, shows variant electrode configurations which can be employed in the method of assay of the present invention, and

Figure 5 is a graph of concentration in attomoles of λ -DNA, immobilised by hybridisation with a target DNA attached to a cellulose-coated magnetic particulate substrate and labelled with an alkaline phosphatase to produce phenol from a phenyl phosphatase substrate against current in micro-amps at a pyrolytic graphite electrode.

Example 1

Figure 1, step 1, shows the addition of a nitrocellulose-treated magnetic particles (MAG)(NITC) to a mixture containing single stranded nucleic acids (NUCA) to form a complex (MAG)-(NITC)-(NUCA).

It will be understood that any nucleic-acid-linkable cycling material can be used, and that cellulosic materials, of which nitrocellulose is only a non-limiting examples, are preferred but not essential. An optional magnetic separation can now take place (as described in more detail below) whereby non-DNA or non-RNA material can be separated from the mixture. This leaves a magnetic particle complex of various nucleic acids to be investigated for the target sequence.

Step 2 shows addition of excess of a probe sequence linked to biotin, which by a hybridisation reaction forms the complex (MAG)-(NITC)-(NUCA)-(PRO)-(BIO) with those nucleic acids containing the target sequence, leaving excess (PRO) - (BIO) unreacted. A magnetic field is applied to retain all complexes containing the (MAG) and therefore those complexes including the target nucleic acid. The excess (PRO)-(BIO) can therefore be washed away without substantial loss of the complex of probe and target nucleic acid. The other (NUCA) species are still present, but not labelled with (PRO)-(BIO).

Step 3, shows addition of excess of a detector system for the biotin marker employed. The detector system in this particle example comprises a conjugate of avidin or streptavidin (AV) and horseradish peroxidase (PER), although other systems can of course be used. Upon

addition of this conjugate to the sample containing the biotinylated probe there is a binding reaction between the avidin and the biotin to link the peroxidase to the target and probe nucleic acid complexes only, i.e. not all nucleic acids present;

Thus the system comprises (MAG)-(NITC)-(NUCA)-(PRO)-(BIO)-(AV)-(PER) plus excess (AV)-(PER).
After allowing time for the reaction to proceed, a magnetic field is applied to retain the large complex and the excess (AV)-(PER) is washed away without disturbing the large complex.

At this stage the assay mixture will still contain much or all of the original nucleic acid which was absorbed onto the coated magnetic particles. However, only those target sequences which are homologous to the probe have formed a duplex with the probe and have therefore complexed the peroxidase. Hence, the concentration of peroxidase in the solution is related directly to the degree of duplex formation and therefore to the degree of homology between the original target nucleic acid and the probe.

Step 4 shows an electrode (E) placed in contact with the resulting suspension containing
(MAG)-(NITC)-(NUCA)-(PRO)-(BIO)-(AV)-(PER). The

H_2O_2, the substrate of the peroxidase, is added together with ferrocene as a suitable mediator compound. The catalytic current generated is proportional to the (AV)-(PER) activity and hence to the concentration of the nucleic acid which has been hybridised to the probe.

At this stage it is possible therefore to obtain a measurement of the target nucleic acid concentration. Furthermore, as the assay mixture still contains much of the original nucleic acid (in a partially purified form) it is possible to probe the assay mixture again for other sequences, by the use of other probes. Unless these additional probes bind to sequences which overlap with those which have already formed a duplex, further hybridisation can occur between the target nucleic acid and the new probe and the analysis may be continued (with a suitable compensation for the background peroxidase activity). As the concentration of the peroxidase can only be represented at the electrode in the presence of peroxide, further probes which employ enzymes different from peroxidase, and therefore show charge transfer in the presence of different substrates, are more usefully employed in the detection of other sequences in the same assay mixture.

Figure 2 shows one form of an apparatus according to the

present invention and for use in the method of the present invention. It consists of two vessels (1 and 2) separated by a magnetic filter (3). Flanged edges (4 and 5) are provided to seal the joint between the vessels. The filter comprises a movable magnetic or magnetisable grid or gauze (6) together with a fixed gauze (7) which latter for ease of operation should preferably be coated with a teflon$_{TM}$ or teflon$_{TM}$-like material. The magnetic gauze (6) can if necessary be used as an electrode; if so it should have a surface of gold or other suitable metal, electroplated or otherwise deposited onto a magnetisable or magnetic gauze support. If the grid or gauze is not to be used as an electrode, it can be coated with a material such as teflon$_{TM}$ and silanised to inhibit absorption. The interior of the vessels should also be silanised before use or made of a suitable material such as polypropylene to prevent absorbtion of materials on to the vessel walls.

The lower vessel (2) is provided with a drain (16) and a suction port (15).

The grid or gauze can be constructed of any permanent magnetic material, such as iron, cobalt or nickel. Alternatively, if windings are placed around the joint, a potentially magnetisable material can become magnetic

on the passage of a current and demagnetised when the current is off.

The flanged edges (4 and 5) may be greased, or provided with O-rings (9) or both. Alternatively, a bayonet-type fitting may be employed. An exterior holding clip or clamp (8) may be provided to ensure a good seal and/or rigidity of the apparatus.

In the embodiment shown in Fig. 2, the upper grid (6) is movable relative to the lower grid by a sliding action. In the figure, the upper grid is shown to be moved by a tag (10). A number of other methods can be used to provide movement such as a rod along the axis of the upper vessel. The tag, however is advantageous in that it may form an electrical terminal if the upper grid (6) is to constitute an electrode. When the upper grid (6) moves relative to the lower materials can be caused to fall from the upper chamber to the lower chamber and be removed from the assay mixture.

Further detail of the sliding action is shown in Figures 2a and 2b. The two grids are open to the passage of materials in Figure 2a but closed to the passage of materials in Figure 2b.

A coil (11) is located about the lower part of the upper vessel and generates a magnetic field when current is

passed through it. (As an alternative, a permanent magnet may be employed, either hand-held or as part of a test-tube rack).

Fig. 3 shows steps of a method of the present invention carried out in an apparatus according to the present invention.

In the first portion of the figure there is shown a vessel in which (MAG)-(NITC)-(NUCA) (by way of example) has already been formed and separated from an earlier mixture or else in which a pure nucleic acid sample is utilised not needing such separation. The biotinylated probe (PRO)-(BIO) is added, in suitable excess. Time is allowed for duplex formation to occur between the (PRO)-(BIO) probe and the target sequences in the (MAG)-(NITC)-(NUCA). During this stage of the reaction, the composite grid 3 i.e. the two grids or gauzes 6 and 7 are closed to the passage of materials from the upper vessel (1) to the lower vessel (2).

In the second drawing of Fig. 3, to remove the excess (PRO)-(BIO) from the assay mixture a magnetic field is applied to the apparatus by the coil (11). Ferromagnetic members (13) may if desired be placed within the field generated by the coil (11) so that the magnetic particles (12) become immobilised upon them and

are held in the upper vessel (11). Alternatively, if a magnetic or magnetisable grid is present the magnetic particles (12) attach themselves to this grid when the field is on.

The grid is then opened to the passage of materials from the upper vessel to the lower vessel. The assay mixture may therefore be washed through by the addition of a suitable fluid from the direction indicated by the letter A in the figure. Excess (PRO)-(BIO) therefore becomes washed out of the apparatus in the direction indicated by the letter B.

In the third portion of Fig 3 the grid (3) is closed again and a suitable buffer solution is placed in the upper chamber (1). The current to the coil (11) (or grid) is switched off. The magnetic particles return into suspension or solution within the upper chamber.

The above description relates to removal of excess (PRO)-(BIO) from the assay mixture. However, other magnetic separation stages i.e. the previous (NUCA)-(NITC)-(MAG) separation or the subsequent separation in which excess detector is removed from the assay mixture, has a like formal protocol.

The coil (11) may if desired also be employed to agitate

the magnetic particles within the upper chamber by the application of a suitably varying magnetic field.

Fig. 4 shows in its three parts and in diagrammatic cross-sections three different electrode configurations suitable for use with the apparatus of the present invention in the final stage of direct electrochemical measurement of the enzyme reaction.

Fig. 4a shows a more or less orthodox three-electrode configuration comprising a working electrode (W), an auxiliary electrode (A) and a reference electrode (R). Further details and examples of electrode configurations of this general type and of a similar, two-electrode, type are given in the patent applications identified above.

Fig. 4b shows a working electrode (W) provided with a winding (14) to enable the electrode itself to become magnetic as and when desired. This has the effect of concentrating on the electrode reagents which are per se magnetic or magnetisable.

Fig. 4c shows the working electrode (W) constitute as one or other component of the grid.

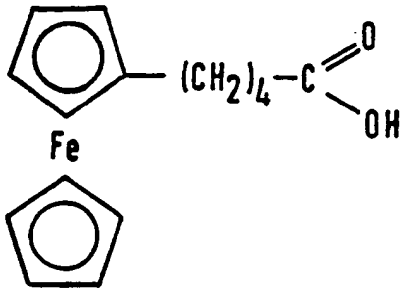
The following example shows stages in the preparation

and properties of a particularly valuable ferrocene derivative which can be utilised in one preferred electrochemical measurement procedure, and which exemplify another aspect of the present invention which consists in a nucleotide labelled with a covalently-linked electoactive species, e.g. a metallocene such as ferrocene; more specifically a ferrocene-labelled -UTP is envisaged. Another aspect of the invention comprises the use of such materials in the assay of nucleic acid sequences.

Example 2

PART I

Preparation of 5-Ferrocenyl Pentanoic Acid



5-Ferrocenyl pentanoic acid was prepared by the procedure published in J.Am. Chem. Soc. (1957) 79, 3420 by Rinehart et al.

PART II

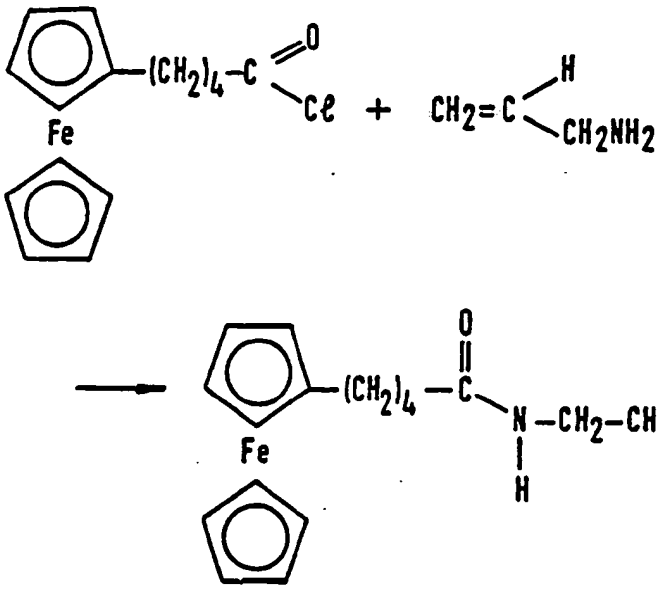
Preparation of N-Propen-2-enyl.5-Ferrocene Pentamide:

$Fc\ (CH_2)_4CONHCH_2CH{=}CH_2$

(i) Preparation of Ferrocenyl Pentanoyl Chloride

$Fc\ (CH_2)_4COCl$

To a solution of 5-Ferrocenyl pentanoic acid in Toluene (500ml) containing pyridine (1.0mL) under dinitrogen atmosphere was added, dropwise, a solution of phosphorous trichloride (PCl_3;2.87 g (1.83mL) 21 mmol). The solution turned cloudy with the formation of a fluffy white precipitate. After completing the addition, the mixture was refluxed for 4 hours. After cooling to ambient temperature, the yellow solution was decanted and the volatiles removed under reduced pressure to give an orange oil. This oil was dissolved in hexane and used in the next step without further purification.



To a vigorously stirred solution of allylamine (1.6 g (2.1mL; 28 mmol) in freshly dried and distilled Tetrahydrofuran (THF) (100 mL) under a nitrogen atmosphere was added dropwise, over a period of 15 minutes, the hexane solution of $Fc(CH_2)_4COCl$. After stirring for 30 minutes the solution was filtered to remove an offwhite precipitate. Removal of volatiles under vacuum gave the title compound as a yellow powdery solid.

Yield 3.9 g (86%). M.Pt 76-78°C.

Mass spectrum: $M.^+/e$ 325 for $FeC_{18}H_{23}NO$

I.R. (Nujol; KBr plates) 3195 cm^{-1}(N-H);1640 cm (C=O); 1550 cm^{-1}(N-H);1110 and 1005 cm^{-1}(C-H) for the ferrocene ring

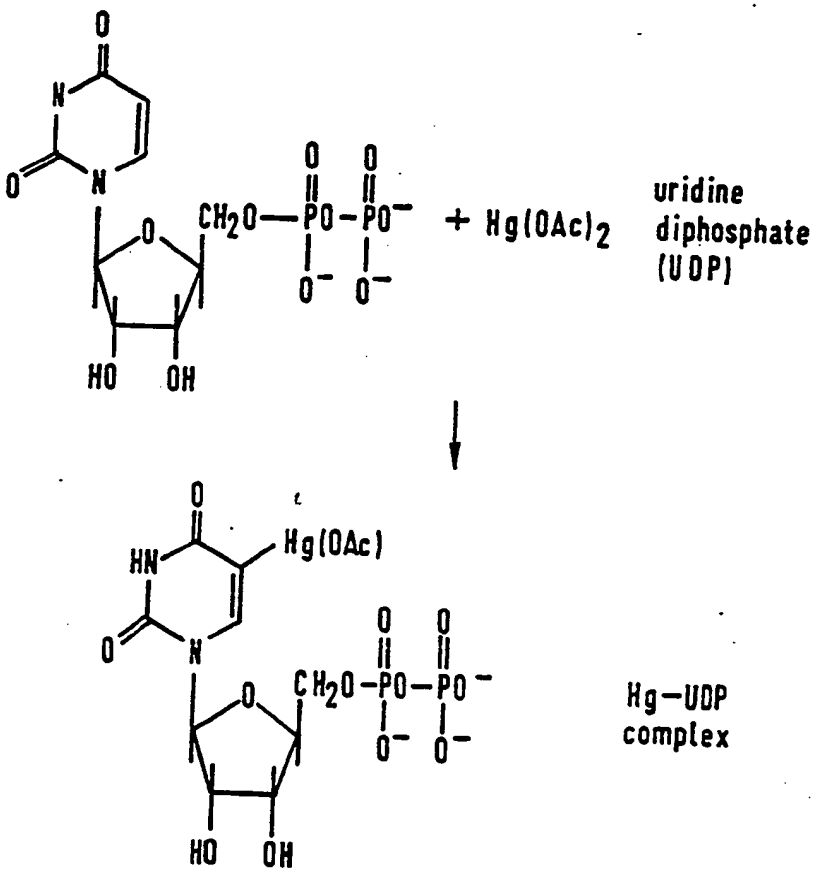
^{1}HNMR($CDCl_3$):5.85(m,1H,C<u>H</u>=CH_2);5.44(S,br,1H,NH) 5.17(m,2H,CH=C<u>H</u>$_2$);4.10(S,5H,C_5H_5);4.05(m,4H,C_5H_4); 3.89(m,2H,allyl C<u>H</u>$_2$);2.36(t,2H,CH_2);2.21(t,2H,CH_2) 1.69(q,2H,CH_2) and 1.53(q,2H,CH_2).

PART III

Preparation of Ferrocene-Uridine Diphosphate Conjugate:

$Fc(CH^2)_4CONH\ CH_2CH=CH=UDP$

i) Mercuration of Uridine Diphosphate at C-5



The uridine diphosphate (UDP) (Sigma) was mercurated at the C-5 position by the literature procedure described by Langer et al Proc. Natl. Acad.Sci. USA (1981),78,6633.

(ii)Preparation of Ferrocenylated - UDP

$$Hg\text{-}UDP + Fc(CH_2)_4CO\cdot NH\cdot CH_2CH=CH_2 \xrightarrow{K_2PdCl_4} Fc(CH_2)_4CO\cdot NH\cdot CH_2CH=CH\text{-}UDP$$

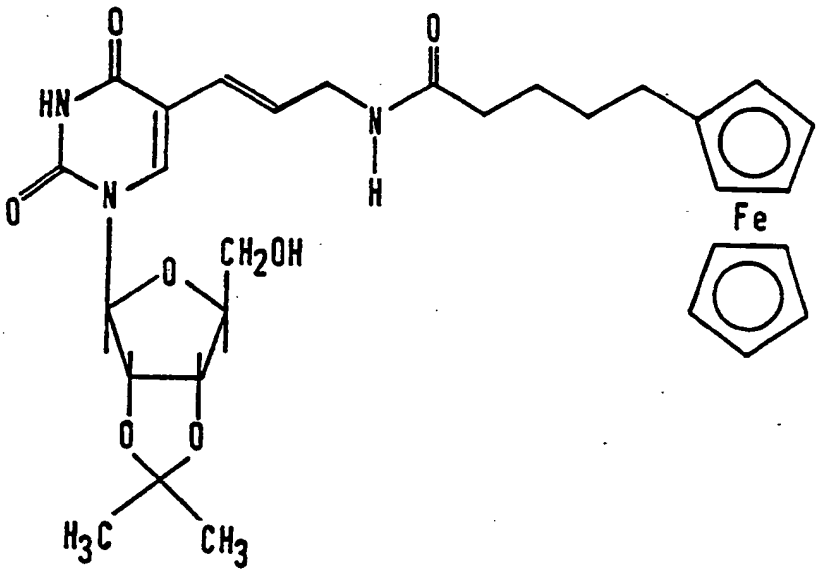
To a solution of mercurated UDP (380 mg; 0.56 mmol) dissolved in 0.1 M sodium acetate (100 mL;pH 5.0) was added a solution of $Fc(CH_2)_4CONHCH_2CH=CH_2$ (360 mg;1.12mmol) in tetrahydrofuran (25mL). Addition of potassium tetrachloropalladate, K_2PdCl_4,(ALdrich); 183 mg (0.56 mmol) dissolved in deionized water (15mL) resulted in an instantaneous formation of a black powder. The mixture was stirred at ambient temperature for 3 days. The black precipitate was removed by centrifugation to give a blue-green supernatant solution which was decanted. The precipitate was washed with further sodium acetate (0.1M;2x25mL). To the combined sodium acetate solution was added three times by volume of ethanol which on cooling to -20°C gave a fine brown precipitate which was collected by centrifugation and

removal of the supernatant and washed successively with with ethanol and diethyl ether and dried (yield; 100 mg).

IR(KBr disc): 3420 cm^{-1} (υO-H) 1680 cm^{-1}(υC=O); 1550cm^{-1}(σN-H);1240cm^{-1}(λP=O);1060$cm^{-1}\lambda$P=O).
Due to the broadness of the above bands, absorptions due to the ferrocenyl group are obscured. However, cyclic voltametric studies confirm the presence of the ferrocene moiety.

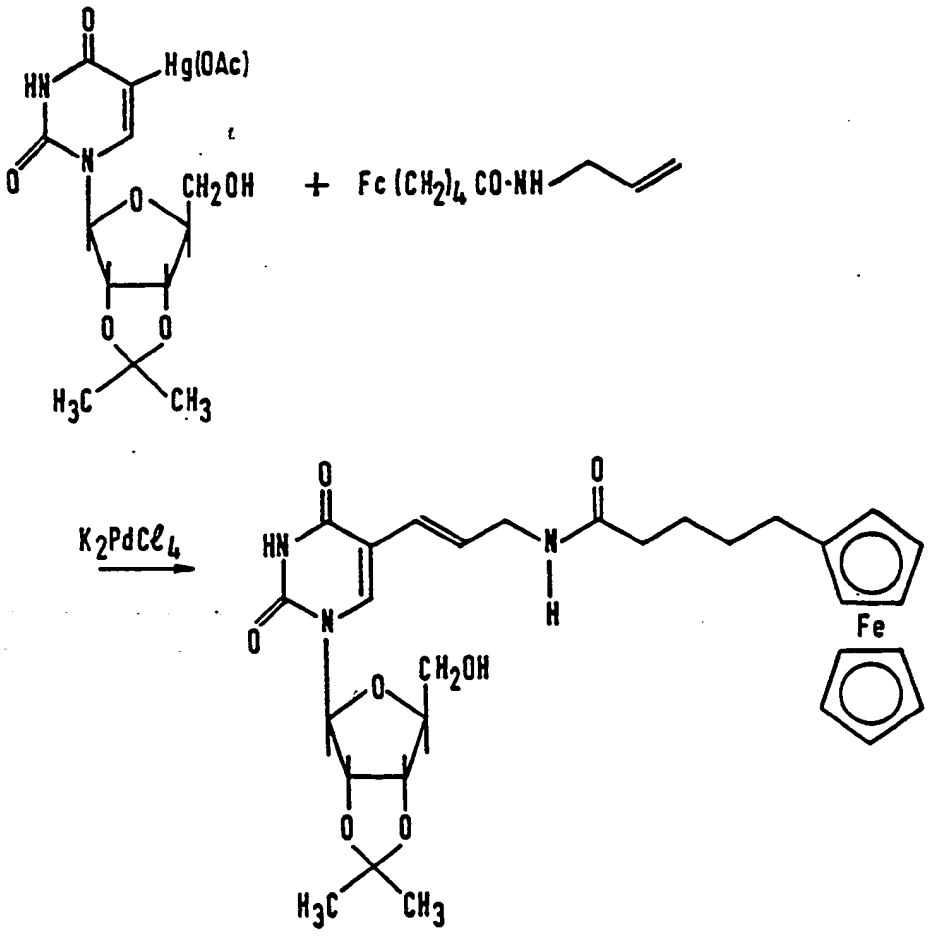
PART IV

Preparation of 2',3'Isopropylidene Uridine-Ferrocene Conjugate



(i) Mercuration of 2',3' Isopropylidene Uridine

To a solution of 2',3' isopropylidene uridine (2.0g; 8.06 mmol) in deionised water (400 mL) was added mercuric acetate (12.84g; 40.28 mmol). The solution was heated at 50°C for 4 hours and cooled in an ice bath. Lithium chloride (1.40 g; 32.22 mmol) was added and the solution extracted with ethylacetate (6x200mL) to remove excess mercuric chloride. The aqueous solution was used in the subsequent step without further purification.



To a solution of mercurated isopropylidene uridine prepared above was added; a solution of $Fc(CH_2)_4CONHCH_2CH=CH_2$(3.17 g;9.8 mmol) dissolved in THF (200 mL). Addition of a solution of K_2PdCl_4 (263 mg 8.1 mmol) in deionised water (25mL) resulted in an instantaneous formation of a very fine black precipitate. The reaction mixture was stirred for 17 hours and filtered to remove the precipitate.To the green filtrate was added L.Ascorbic acid (1.55 g) together with diethylether (500mL). The mixture was vigorously shaken and the organic layer separated from the colourless aqueous layer. The ether phase was dried over magnesium sulphate and removal of the volatiles gave an orange oil.

The product was further purified by column chromatography on silica with acetone-ether (1:1) as eluant. The desired compound eluted as a major yellow band after two minor yellow components and forms an orange oil on removal of volatiles, which subsequently crystallised to a yellow solid on standing at room temperature. This was identified as ferrocenylated 2'-3' isopropylidene uridine conjugate.

Melting point 60-62°C

I.R.(KBr disc) 3300 cm^{-1}(νO-H); 3090 cm^{-1}(μ N-H)
1725 and 1640 cm^{-1} (ν C=O);1550 cm^{1} (σ N-H);
1110 and 1005 cm^{-1}.δ(C-H ferrocene)

^{1}H NMR ($CDCl_3$)

σ9.80 (S,1H,NH); 5.87 (S,1H,NH); 6.16 (m,1H); 5.31 (S,1H,H-6); 4.17 (d,1H); 4.09 (S,5H,C_5H_5); 4.05 (m,4H,C_5H_4); 3.54 (,2H); 2.74 (t,2H,CH_2); 2.4-2.2 (m); 2.21 (S,3H,Me); 2.14 (t,2H,CH^2); 1.66(m,2H,CH_2) 1.62(s,3H,Me;1.50(m,2H,CH_2);0.87(m,1H)

Example 3

This example shows a variant procedure and use of a different electrochemical measurement system.

Materials

All chemicals, unless specifically stated otherwise, were obtained from BDH Ltd. Streptavidin and alkaline phosphatase were obtained from BRL Ltd.

Methods

(a) Cellulose-coated magnetic particles were prepared as described in the literature (Pourfarzaneh et al, Methods Biochem Anal. 28, 267-295 1982.

(b) Magnetic particles were derivatised by reaction with 1,4-butanediol diglycidyl ether/2-aminothiophenol (APTE)as described by Bunemann et al Nucleic Acids Research 10 7163-7180. 1982.

(c) The APTE-matrix was activated by conversion to the corresponding diazophenylthioether (DPTE)-matrix as described by Bunemann et al (see above). The diazo-matrix was prepared immediately prior to the immobilisation of DNA.

(d) Coupling of denatured DNA (as a target material) to the DPTE-support was achieved in the following manner. λ DNA was suspended in 1xSSPE buffer and denatured by the addition of DMSO to produce an 80% v/v mixture of DMSO in buffer. Sonicated salmon sperm DNA was added as carrier to produce a final concentration of 1 mg/ml. The DNA solution (1 ml) was then added to the wet DPTE material (2 g) and processed as described by Bunemann et al, see above

(e) λDNA was biotinylated (to provide a probe material) using nick-translation kit (purchased from BRL Ltd) in the presence of biotin-11-UTP. High molecular weight DNA was purified from unincorporated material by chromatography on a Nensorb cartridge (Dupont). The DNA solution was evaporated to dryness and th pellet was

reconstituted in 1xSSPE buffer at 37° for 20 min.

(f) Hybridisations were carried out in polypropylene vials, sealed in polypropylene bags, submerged in a shaking water bath. λDNA samples linked to the magnetic support were pre-incubated at 65°C for two hours in a cocktail which consisted of 4 x SSPE, 6 x Denhardt's solution, 300µg/ml denatured salmon DNA and 0.1% w/v SDS (1 ml).

The pre-hybridisation mix was removed and replaced with 1 ml of hybridisation cocktail consisting of 4 x SSPE, 2 x Denhardt's solution, 200 µg/ml denatured salmon sperm DNA, 0.1% w/v SDS, 10% w/v dextran sulphate in the following buffers:

(1) 2 ml of 2 x SSC (3 x 5 min at 20°C)

(2) 2 ml of 0.2 x SSC (3 x 15 min at 65°C)

Unbound probe was removed by attraction of the magnetic particles using equipment as shown above, and suitable washing procedure.

(g) Detection of biotinylated probes

Binding of streptavidin and poly alkaline phosphatase was conducted essentially as described in the dot-blot procedure of Chan et al (1985) see above.

(1) Firstly, the magnetic particles were washed at

22°C for 90 min in 1 ml blocking buffer (100 mM NaCl, 100 mM Tris/HCl, 3 mM $MgCl_2$, 0.5% $^{v}/v$ Tween 20, at pH 7.5), magnetic separation as before also being utilised as a separation technique.

(2) The blocking buffer was replaced with 1 ml of a solution of streptavidin (2 µg/ml in above buffer, except that Tween 20 was at 0.05% $^{v}/v$); the incubation was for 10 min at 22°C.

(3) The support was washed 3 x (15 min each) with blocking buffer and finally resuspended in 1 ml of biotinylated poly alkaline phosphatase solution (1 µg/ml in 0.05% $^{v}/v$, Tween 20/blocking buffer). Incubation was for 10 min at 22°C. Thus, the enzyme became attached, via biotin, to the streptavidin, itself biotin-linked to the hybridisation product.

(4) The matrix was then washed to remove unbound phosphatase, in the following manner; 3 x 1.5 ml blocking buffer (5 min each at 22°C), followed by 2 x 1.5 ml developing buffer (10 min each at 22°C), the magnetic procedures again being utilised to retain the desired material. Developing buffer consisted of 100 mM NaCl, 160 mM Tris/HCl, 10 mM $MgCl_2$, pH 9.6.

(5) Alkaline phosphatase bound to the magnetic

matrix was finally detected electrochemically by resuspending the supporting material in 1 ml of developing buffer which contained 5 mM phenyl phosphate, and incubating at 22°C for 10 min. The supernatant was decanted and transferred to a standard three electrode cell (saturated calomel reference, platinum counter and pyrolytic graphite working electrode). Phenol produced by the action of alkaline phosphatase was oxidised at the working electrode which was poised at +600 mV, and the resulting current versus time transients were recorded.

Figure 5 shows the currents obtained when magnetic particles containing various amount of immobilised DNA were probed using the protocols described in the methods section. The results demonstrate the sensitivity of the detection system employed in that 1 attomole of DNA was readily detected above background levels. Furthermore, in the range examined, the current response is directly proportional to the amount of immobilised DNA on the magnetic support. The simple one step hybridisation protocol described above thus has the potential to detect DNA sequences present at the single copy level in mammalian DNA and to allow the quantitation of multiple copy gene sequences. The system can be extended and modified to produce further configurations, for example;

(a) Any marker enzymes that can be either biotinylated and/or covalently linked to streptavidin can be used provided that they produce electrochemically active products (i.e. glucose oxidase, horse radish peroxidase).

(b) The immobilisation of target DNA onto the magnetic support could be circumvented by using a 'sandwich' type procedure, as described previously in the literature by Ranki et al Gene 21, 77-85 (1983) and more recently by Langdale and Malcolm, Gene 36, 201-210 (1985) in which two non-overlapping restriction fragments of the gene of interest are utilised. Fragment A is covalently linked to the magnetic support and fragment B is labelled with biotin. The presence of a contiguous DNA/RNA sequence in a crude DNA mixture that is capable of hybridising to both A and B will effectively result in the biotinylation of the magnetic support (detected as described above).

(c) The ease of handling of the magnetic solid phase support and the convenience and speed of the washing steps facilitated by its paramagnetic nature mean that the system could readily be incorporated into an automated analytical device.

CLAIMS

1. An entity including a single or double-stranded nucleic acid sequence linked to a magnetic or magnetisable substance.

2. An entity as claimed in Claim 1 in which the nucleic acid is single-stranded.

3. An entity as claimed in Claim 2 provided in particulate form.

4. An entity as claimed in Claim 3 in which the nucleic acid is of unknown composition for investigation as containing a target sequence.

5. An entity as claimed in Claim 3 in which the particles are ferromagnetic.

6. An entity as claimed in Claim 5 in which the particles are coated with a material to which the nucleic acid attaches.

7. An entity as claimed in Claim 6 in which the coating material is a cellulose derivative.

8. An entity as claimed in Claim 7 in which the

derivative is nitro-cellulose.

9. The use of particles of a magnetic or magnetisable substance, coated with a material capable of attachment to a DNA or RNA single-strand material, in a magnetic separation under the influence of an applied magnetic field for the purpose of separating (a) such nucleic acids from a mixture of materials or (b) attached nucleic acids hybridised with a nucleic acid containing a complementary sequence from excess of the unattached complementary nucleic acid or (c) such attached and hybridised materials from an excess of an enzyme marker, or of a material convertible to an enzyme marker, reactive therewith.

10. A method of separation of single-stranded nucleic acid materials from a complex mixture containing such materials, in which the mixture is treated with magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and the particles and attached nucleic acid materials are thereafter separated from the other components, at least in part, by a magnetic field.

11. A method for the detection or quantification of the presence of a target sequence of nucleic acid units

or number of such sequences , in a single-stranded DNA or RNA material or mixture of such materials, by contact with a probe DNA or RNA sequence of a predetermined level of homology to the target sequence, followed by separation of the materials for detection or quantification of the probe/target hybridisation reaction; in which the probe sequence or the target sequence is a single-stranded nucleic acid sequence linked to a magnetic or magnetisable material in the form of particles having a coating to which a nucleic acid single strand material becomes permanently attached, and in which separation is at least in part effected by a magnetic field.

12. A method for the detection or quantification of the presence of a target sequence of nucleic acid units, or number of said sequence, in a single-stranded RNA or DNA material, or mixture of such materials, in the presence of a complex liquid mixture containing inter alia such DNA or RNA single-stranded materials, comprising the steps of:

(i) contacting the complex liquid mixture with a magnetic or magnetisable material in the form of particles having a coating to which any nucleic acid single-strand RNA or DNA material becomes permanently attached, and thereafter separating the magnetic particles from the mixture, at least in

part, by a magnetic field,

(ii)contacting the separated DNA/RNA material linked to the magnetic or magnetisable particle with a probe single-stranded DNA or RNA sequence to detect or quantify by a hybridisation reaction the presence of the target sequence on said permanently attached DNA/RNA magnetically separated material, and thereafter separating the magnetic or magnetisable material from unbound probe sequence, at least in part, by a magnetic field,

prior to assay of the probe/target bound species.

13. A method as claimed in Claim 10, 11 or 12 in which the coating is a cellulose derivative.

14. A method as claimed in Claim 10, 11 or 12 in which the coating is nitro-cellulose.

15. A method as claimed in Claim 12 in which assay is effected by linkage of the probe sequence with compound A of a pair of compounds A and B which themselves react with a specific binding reaction, whereby B can be incorporated into the bound probe and utilised as a marker, or to attach a marker, for subsequent assay.

16. A method as claimed in Claim 15 wherein compound A

is biotin and compound B is avidin or streptavidin linked to an enzyme th ffect of which on a substrate is used for the subsequent assay.

17. A method as claimed in Claim 16 in which the enzyme action is detected electrochemically and the enzyme is any redox enzyme more particularly a dehydrogenase, or a enzyme which degrades a large molecule such as α-amylase, an RNA-ase or a DNA-ase.

18. A method as claimed in Claim 17 in which the electrochemical activity of the enzyme is detected with use of a mediator compound.

19. A method as claimed in Claim 18 in which the mediator is a metallocene, especially a ferrocene.

20. A method as claimed in Claim 19 in which the enzyme/substrate system is peroxidase/H_2O_2 or glucose oxidase/glucose.

21. A method for detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a cellolose

derivative (DCEL) whereby single-stranded nucleic acids (NUCA) become permanently attached thereto, and thereafter separating the magnetic particles from the mixture
by a magnetic field,
(ii) contacting the (NUCA)-(DCEL)-(MAG) material thus separated with a single-stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the (NUCA)-DCEL)-(MAG) particles possessing a complementary target sequence will react, with hybridisation to give the complex
(BIO)(-PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field
(iii) contacting the separated materials with avidin or streptavidin (AV) linked with a peroxidase enzyme (PER) to form the complex
(PER)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field, and
(iv) bringing the said magnetically separated materials carrying the said complex into contact with hydrogen peroxide and a ferrocene, whereby the terminal (PER) group on the complex causes the hydrogen peroxide to react so that the ferrocene transfers charge to or from a contacting electrode

to feed a readout signal.

22. A method for the detection of a target sequence of nucleic acid units in a single stranded DNA or RNA material, or mixture of such materials, in a liquid mixture, comprising the steps of:

(i) contacting the liquid mixture with particles of magnetic material (MAG) coated with a derivatised cellulose (DCEL) capable of permanently attaching thereto single-stranded nucleic acids, and thereafter separating the magnetic particles from the mixture by a magnetic field,

(ii) contacting the (NUCA)-(DCEL)-(MAG) material thus separated with a single stranded nucleic acid probe sequence (PRO) chemically linked with biotin (BIO) whereby only those nucleic acids on the (NUCA)-(DCEL)-(MAG) particles possessing a complementary target sequence will react with hybridisation to give the complex (BIO)-(PRO)-(NUCA)-(DCEL)-(MAG), and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iii) contacting the separated materials with avidin or streptavidin (AV) to form the complex (AV)-(BIO)-(PRO)-(NUCA)-DCEL)-(MAG) and thereafter separating the magnetic particles from the mixture by a magnetic field,

(iv) thereafter binding to the (AV)-groups of the complex using biotin (BIO) a marker enzyme (ENZ), and (v) bringing the resultant (ENZ)-(AV)-(BIO)-(PRO)-(NUCA)-(DCEL)-(MAG) into contact with a suitable substrate for the said enzyme and measuring the enzyme reaction electrochemically.

23. A method as claimed in Claim 21 or 22 in which the cellulose derivative (DCEL) is an nitrocellulose or aminophenylthioether linkage, activated to the corresponding diazo compound for immobilisation of the single-strand (NUCA).

24. A method as claimed in Claim 22 in which the (AV) group is itself biotin-linked with an alkaline phosphatase utilising phenyl phosphate as a substrate conversible to phenol.

25. Apparatus for use in a method of assay as defined in Claim 10, 11 or 12 comprising an upper vessel, a lower vessel selectively placeable in liquid flow communication therewith, and aselectively applicable magnetic element in said upper vessel.

26. Apparatus as claimed in Claim 25 in which the magnetic element is a magnetisable or magnetic grid element having two layers relatively movable to provide

either separation or communication.

27. Apparatus as claimed in Claim 25 in which the magnetic element is an electrode for subsequent use in the electrochemical determination of anenzyme reaction.

28.A nucleotide labelled with a covalently-linked electroactive species.

29. A ferrocene-labelled nucleotide.

30. A ferrocene-labelled UTP.

31. The use of a nucleotide material labelled as claimed in claim 28, 29 or 30 in the assay of nucleic acid sequences.

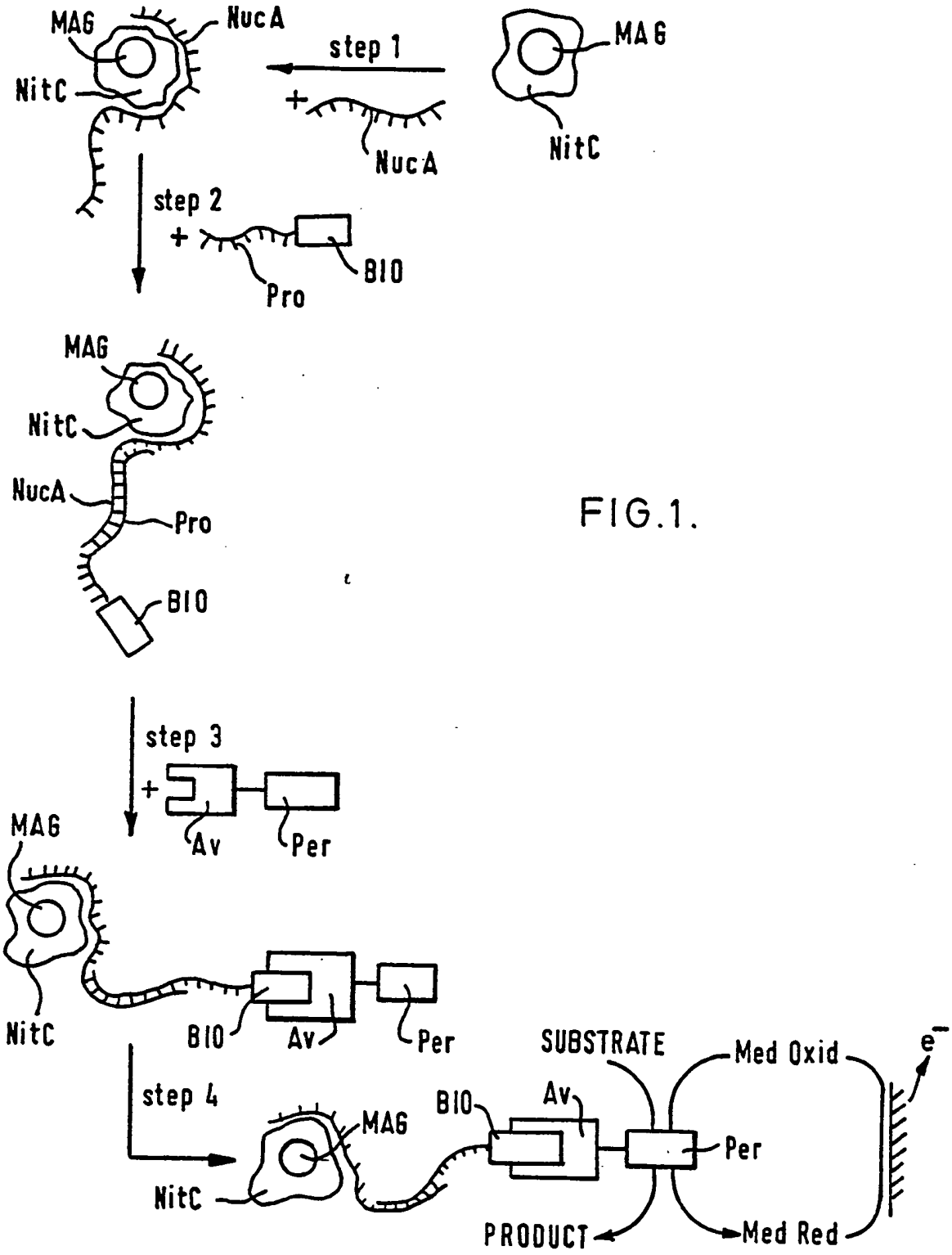


FIG.1.

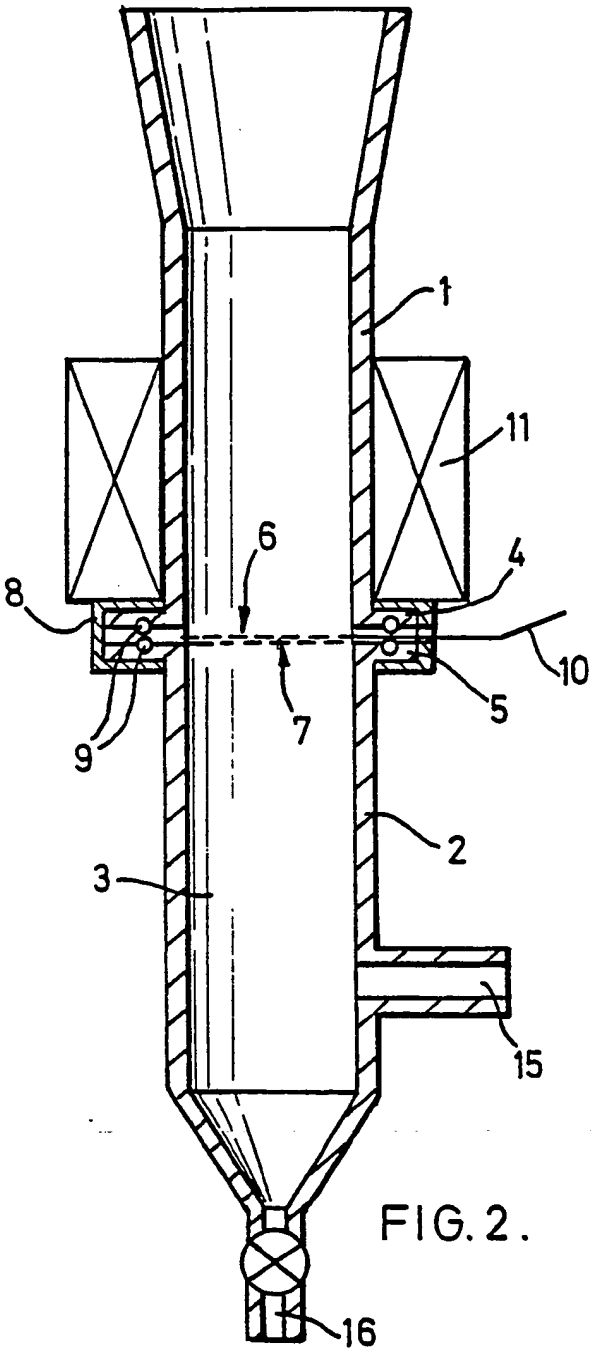


FIG. 2.

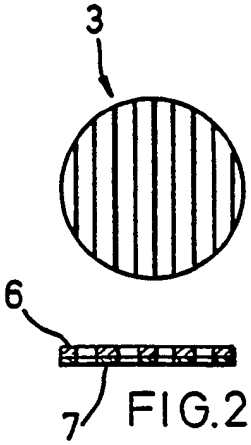


FIG.2a.

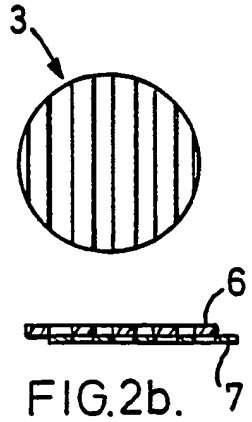


FIG.2b.

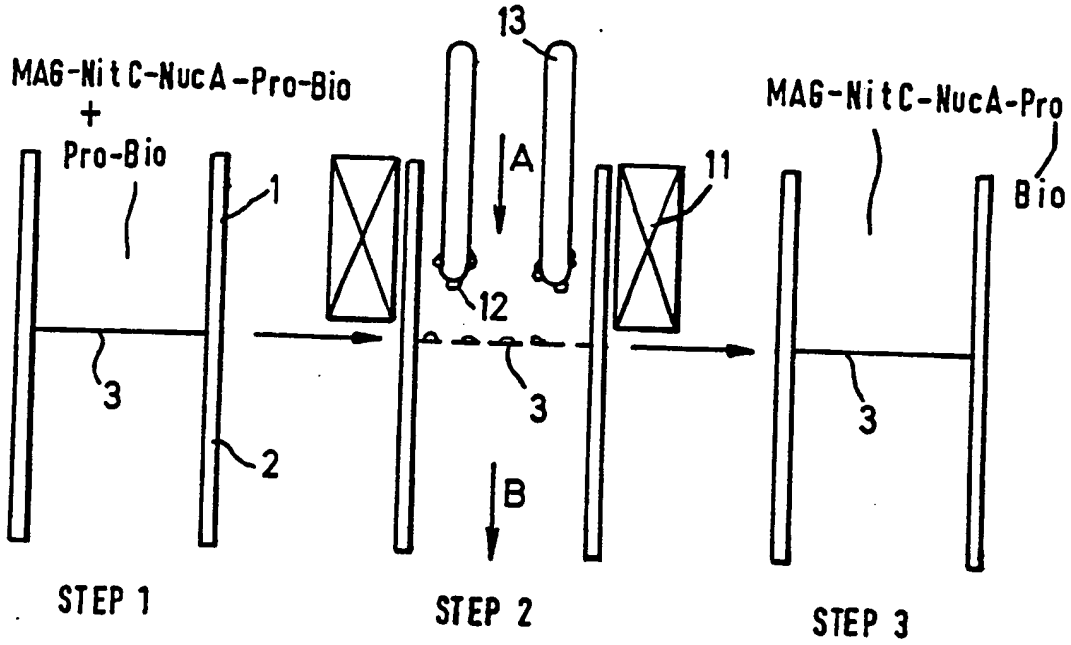

MAG-NitC-NucA-Pro-Bio
+
Pro-Bio
1
2
3
13
A
12
B
11
MAG-NitC-NucA-Pro
Bio
STEP 1
STEP 2
STEP 3

FIG.3.

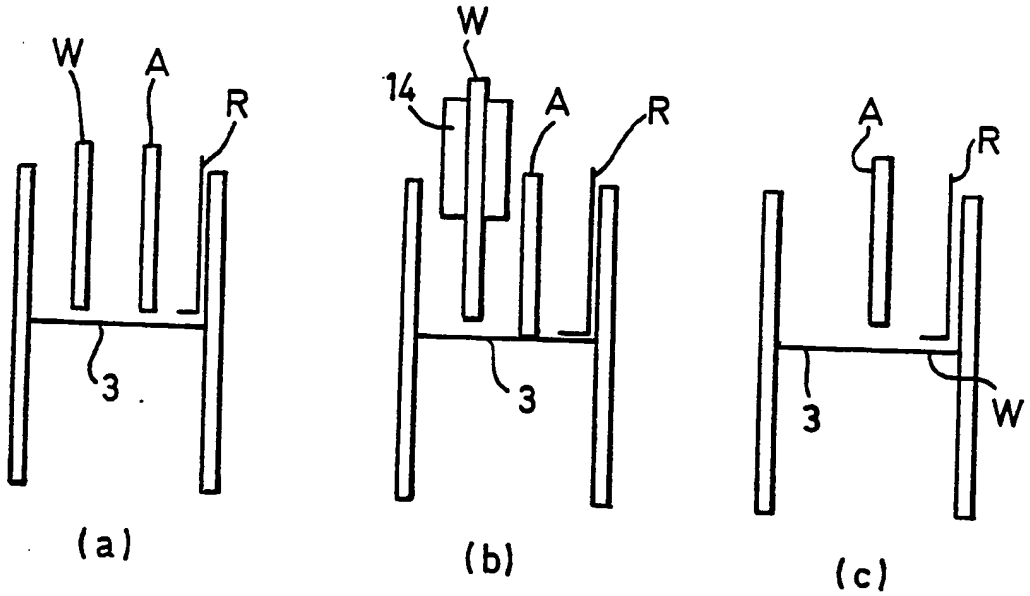

W
A
R
3
14
(a)
(b)
(c)

FIG.4.

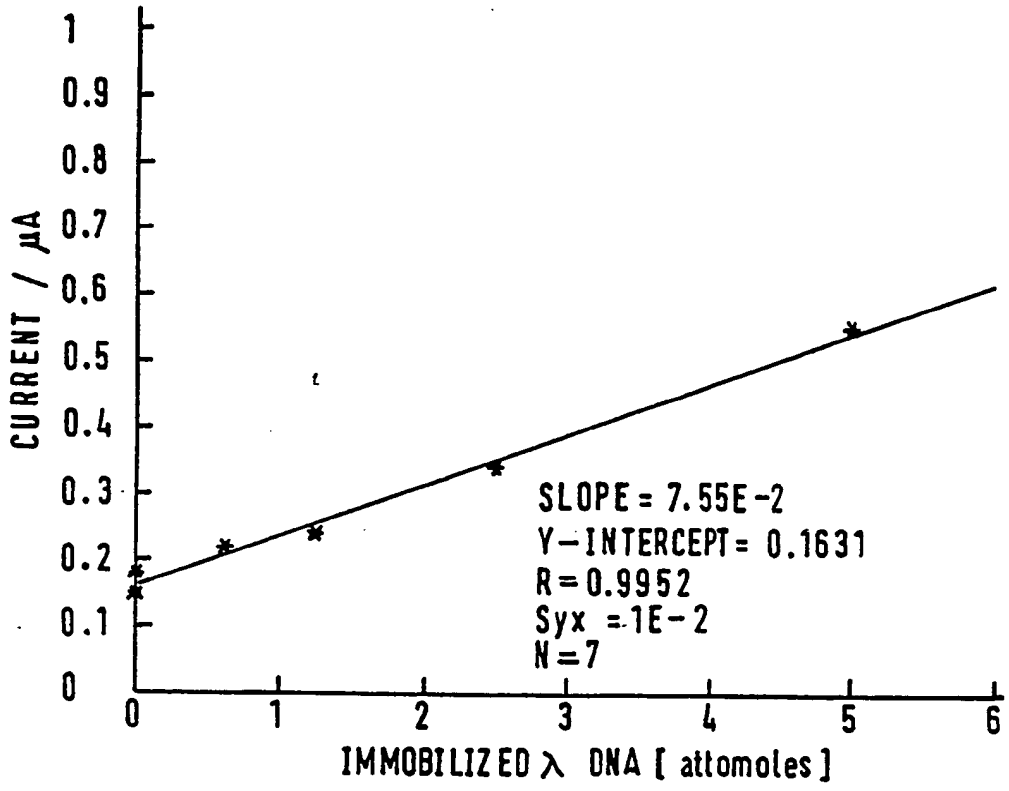

CURRENT / μA
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
IMMOBILIZED λ DNA [attomoles]
SLOPE = 7.55E-2
Y-INTERCEPT = 0.1631
R = 0.9952
Syx = 1E-2
N = 7

FIG. 5.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 86/00174

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 Q 1/68; 1/00; G 01 N 33/50; 33/48; C 07 H 19/067

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 Q; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	Chemical Abstracts, vol. 101, no. 17, 22 October 1984 (Columbus, Ohio, US) J. Langdale et al.: "Magnetic DNA", see page 349, abstract no. 147252w, & Biochem. Soc. Trans. 1984, 12(4), 693-4, see the whole abstract	1-24
P,Y	GB, A, 2152664 (SERONO DIAGNOSTICS LTD.) 7 August 1985 see abstract; pages 2-3; claims 1-23	1-24
A	EP, A, 0030087, (TECHNICON INSTRUMENTS COMPANY) 10 June 1981	
Y	EP, A, 0125995 (ADVANCED MAGNETICS, INC.) 21 November 1984 see abstract; pages 32,33	1-24
P,Y	EP, A, 0149339 (GENETICS INTERNATIONAL INC.) 24 July 1985 see abstract; claims 1-6,14-42	17-22

./.

* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th June 1986	-7 JUL 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION C NTINUED FR M THE SECOND SHEET

Y	EP, A, 0125139 (GENETICS INTERNATIONAL INC) 14 November 1984 see abstract; examples 3a,3b,3c; claims 1,15	17-22
Y	EP, A, 0070687 (STANDARD OIL COMPANY) 26 January 1983 see abstract; page 4, lines 4-28	3,6-8

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Claims 1-24 : Magnetic nucleic acid particles; use in the assay for nucleic acid.
Claims 25-27: Apparatus for use with magnetic particles.
Claims 28-30: Ferrocene labelled nucleotides per se.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-24

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 86/00174 (SA 12721)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/09/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publicatic date
GB-A- 2152664	07/08/85	None		
EP-A- 0030087	10/06/81	None		
EP-A- 0125995	21/11/84	JP-A-	60001564	07/01/85
		US-A-	4554088	19/11/85
EP-A- 0149339	24/07/85	EP-A-	0125139	14/11/84
		AU-A-	2775384	08/11/84
		WO-A-	8502627	20/06/85
		AU-A-	3832985	26/06/85
		JP-T-	61500706	17/04/86
EP-A- 0125139	14/11/84	AU-A-	2775384	08/11/84
		EP-A-	0125136	14/11/84
		EP-A-	0125137	14/11/84
		EP-A-	0125867	21/11/84
		AU-A-	2775484	08/11/84
		JP-A-	60017345	29/01/85
		JP-A-	60017346	29/01/85
		JP-A-	60017347	29/01/85
		JP-A-	60017360	29/01/85
		AU-A-	2775184	31/01/85
		AU-A-	2775284	31/01/85
		WO-A-	8502627	20/06/85
		EP-A-	0149339	24/07/85
		AU-A-	3832985	26/06/85
		JP-T-	61500706	17/04/86
		EP-A-	0127958	12/12/84
EP-A- 0070687	26/01/83	JP-A-	58040099	08/03/83
		CA-A-	1180647	08/01/85

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82

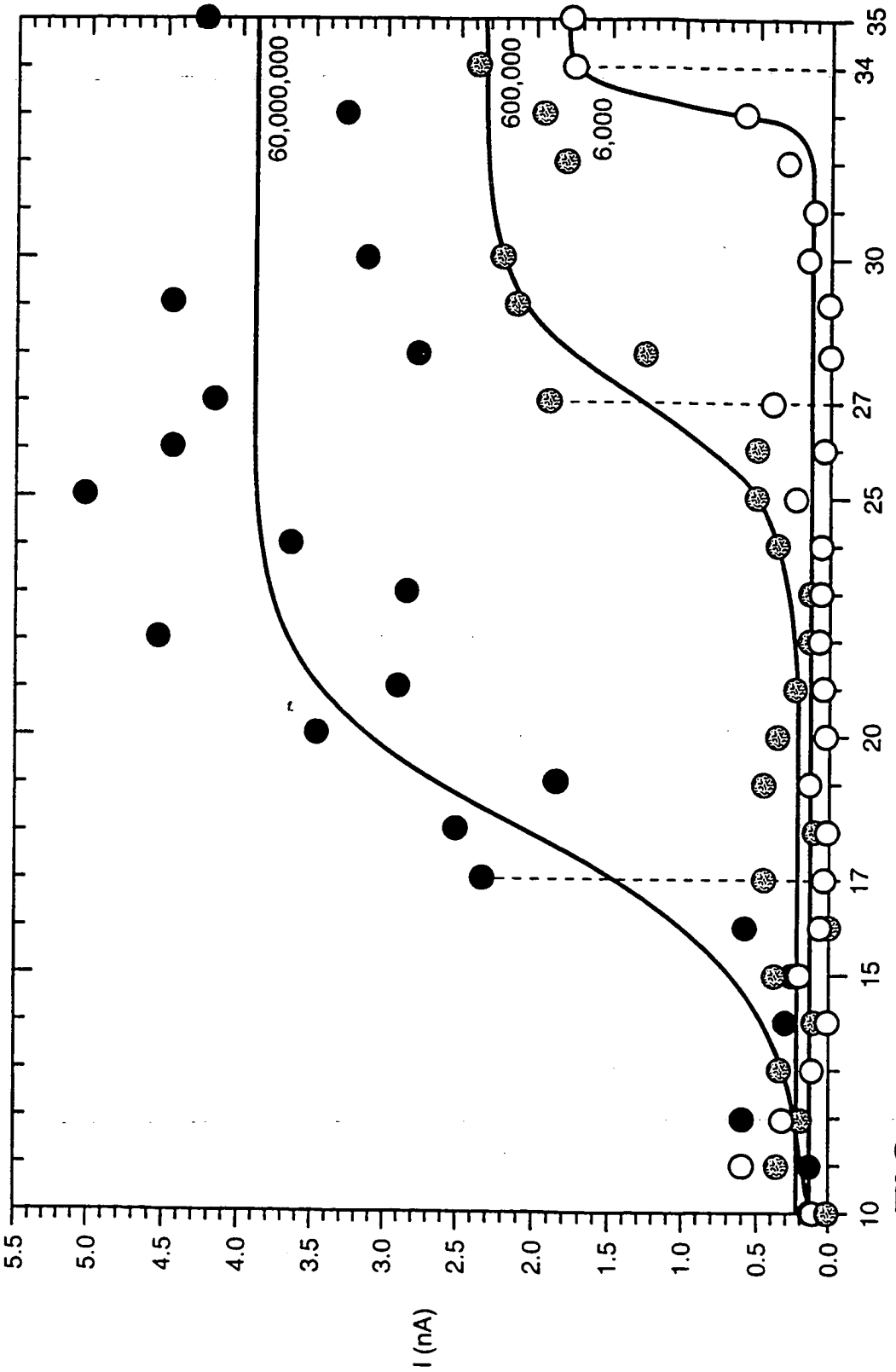


FIG._26

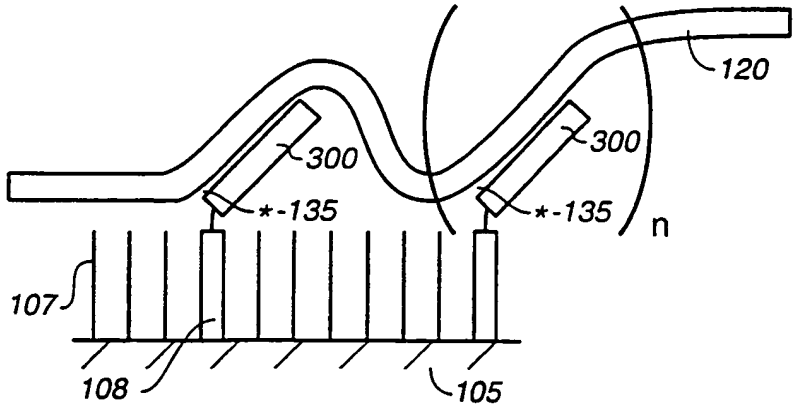


FIG._27A

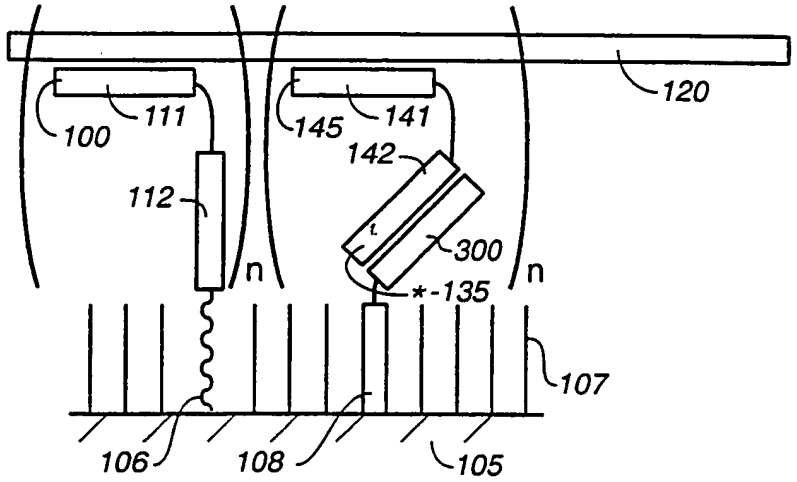


FIG._27B

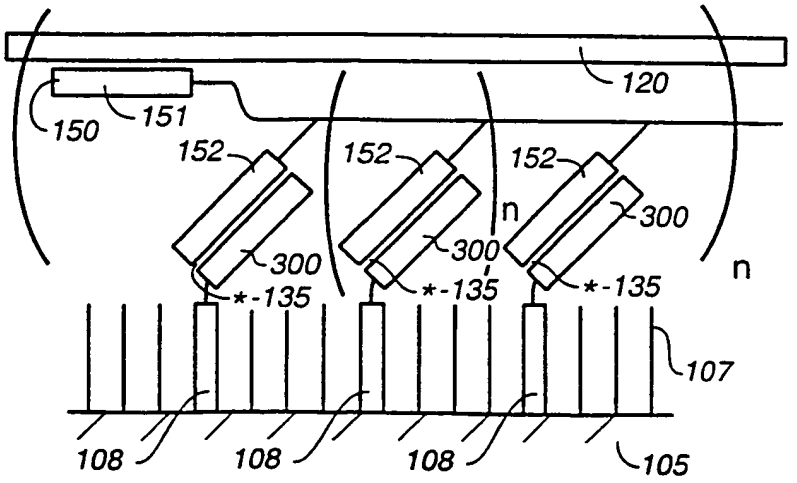


FIG._27C

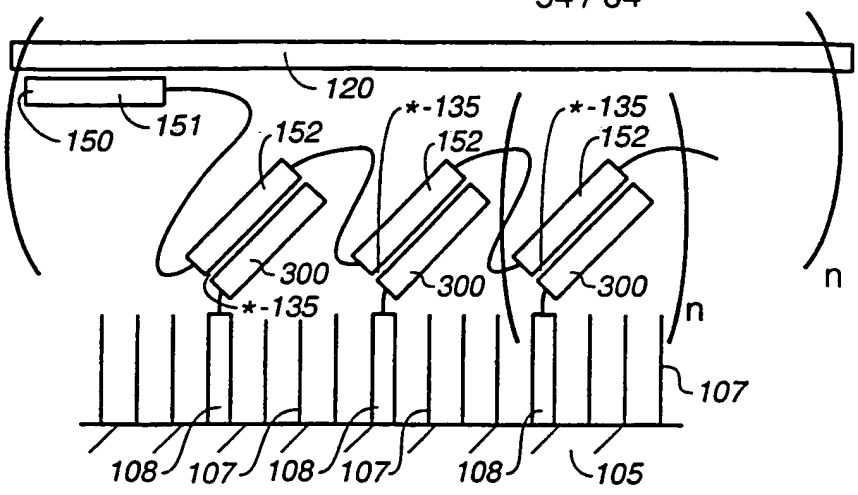


FIG._27D

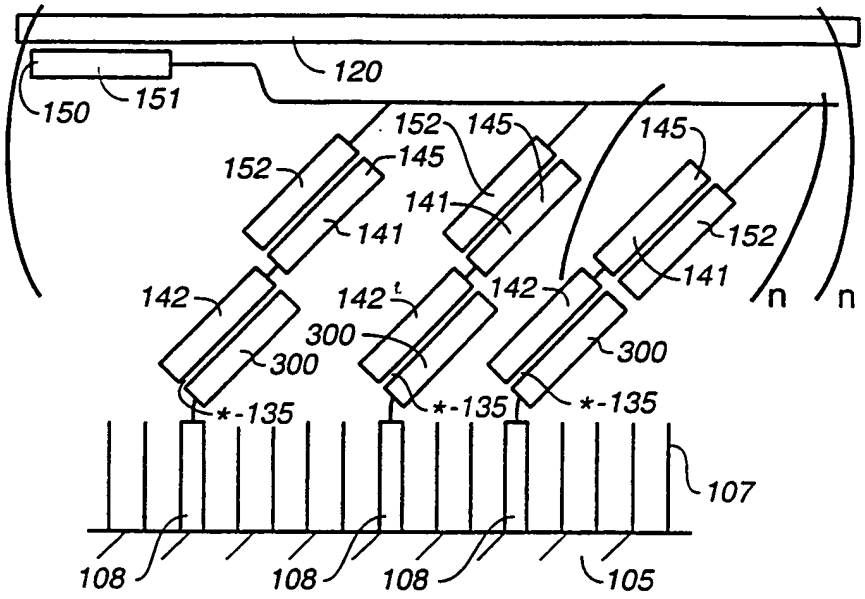


FIG._27E

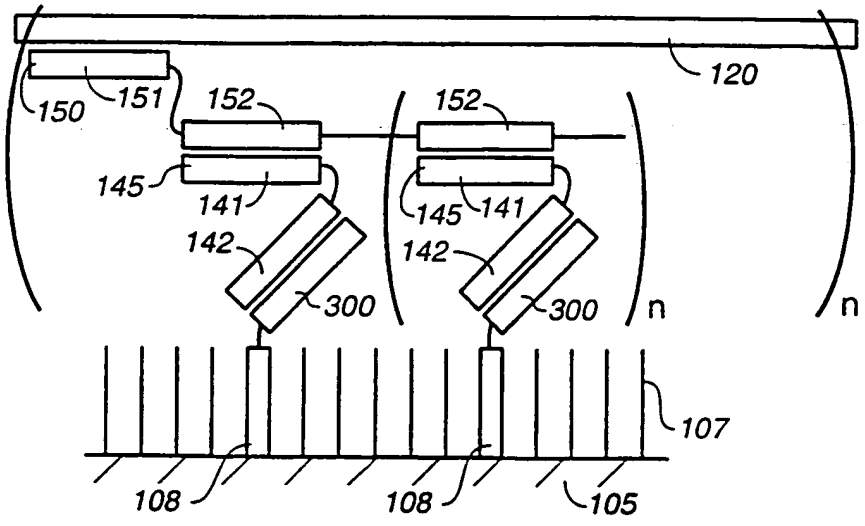


FIG._27F

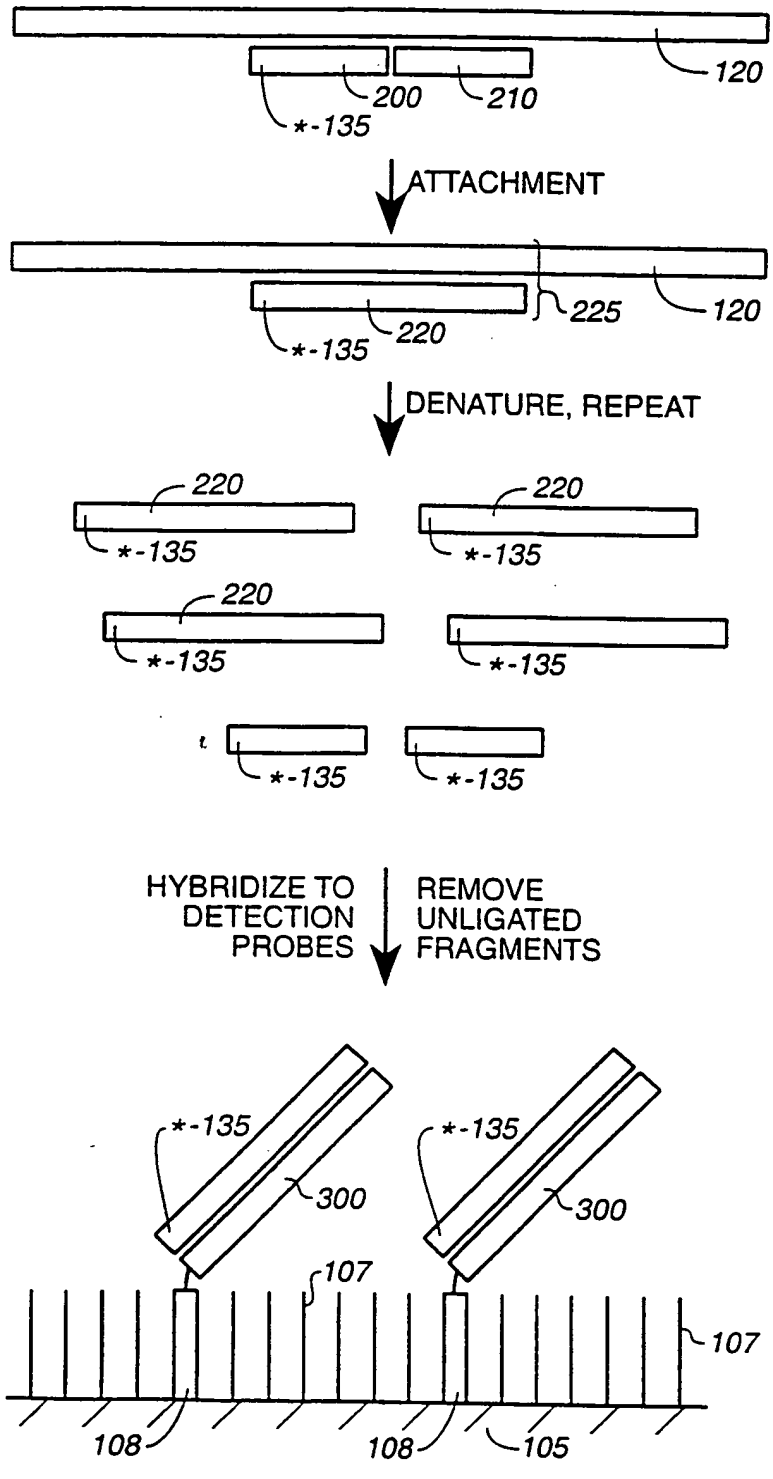
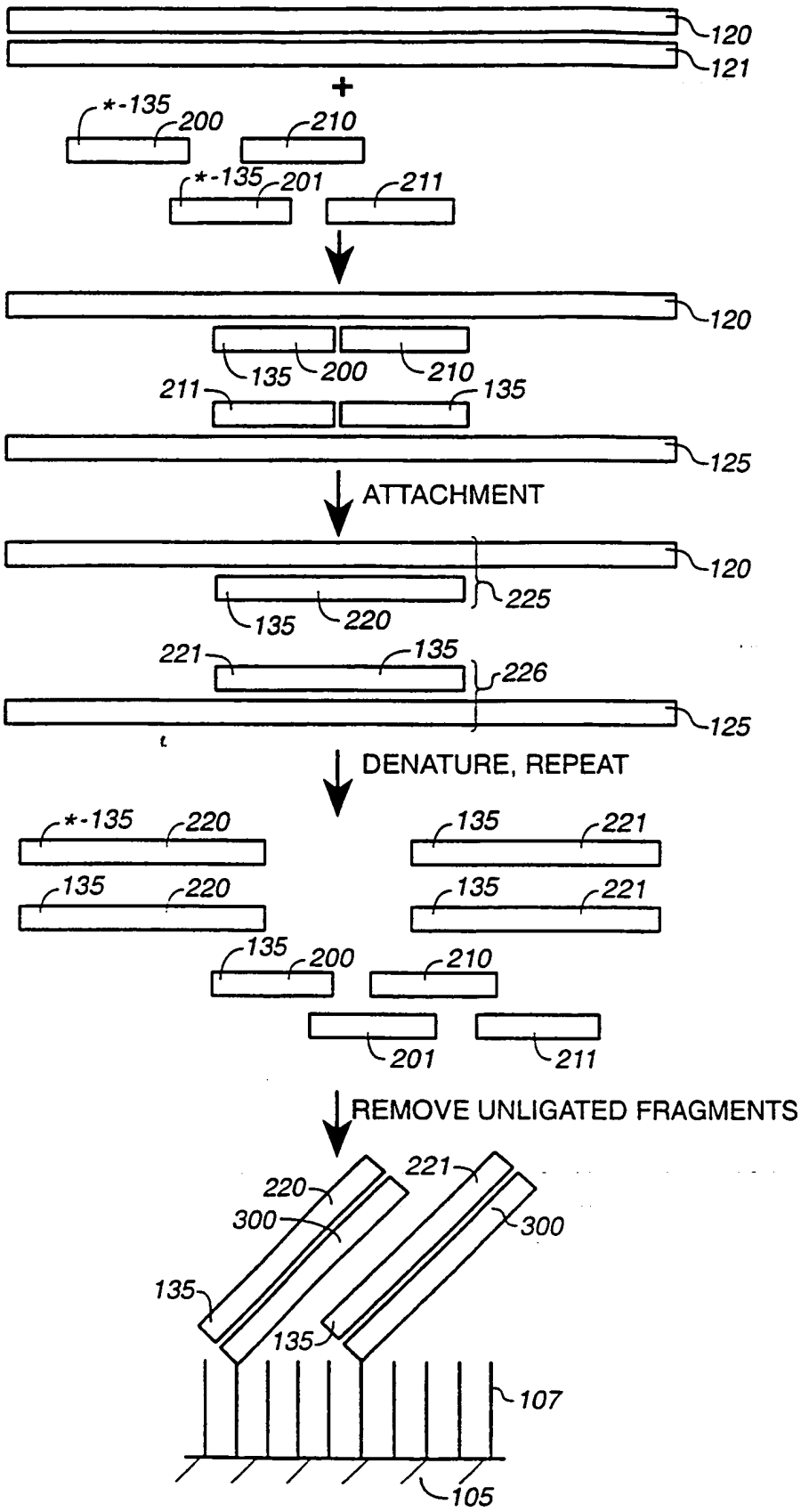


FIG._28



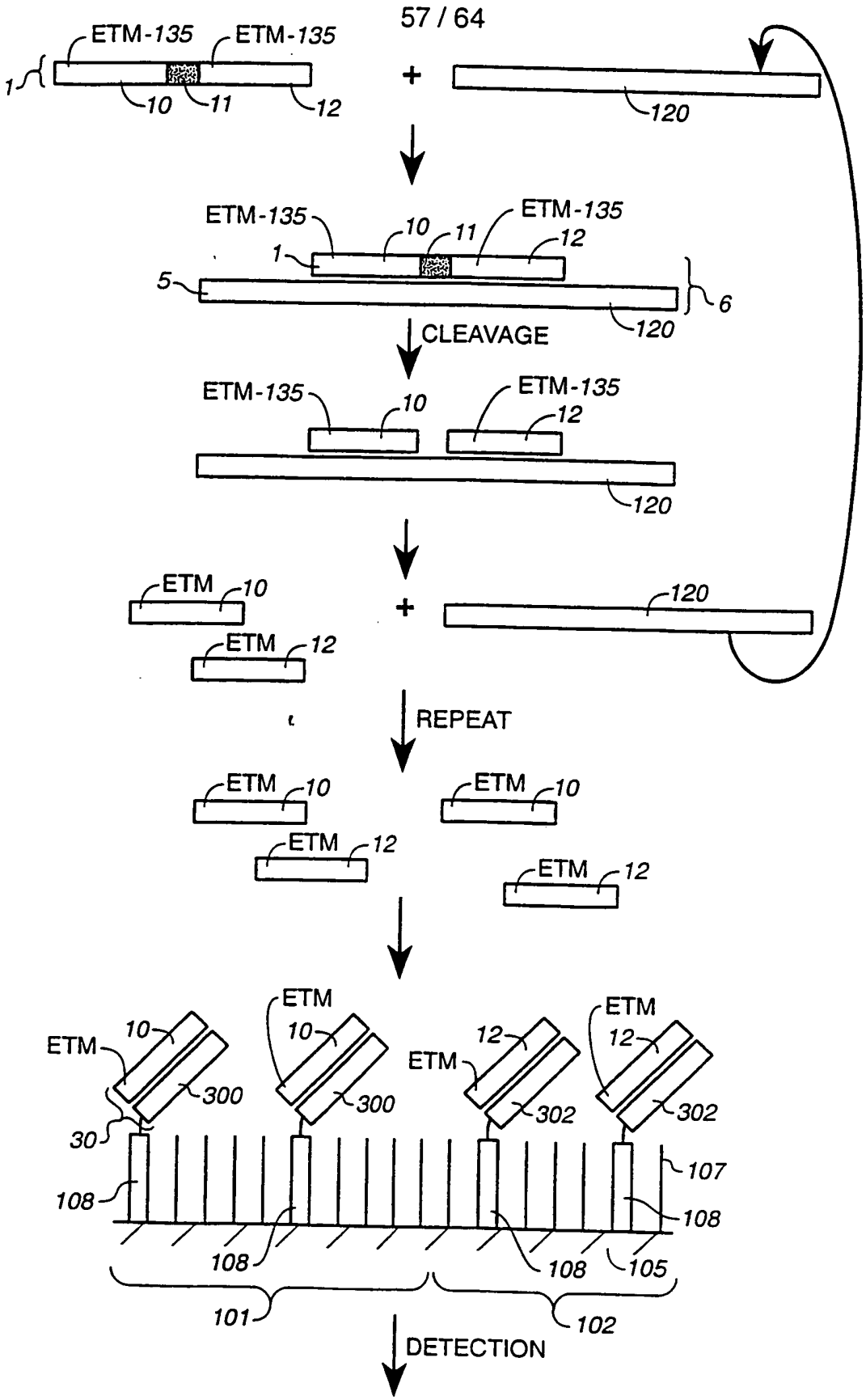


FIG._30

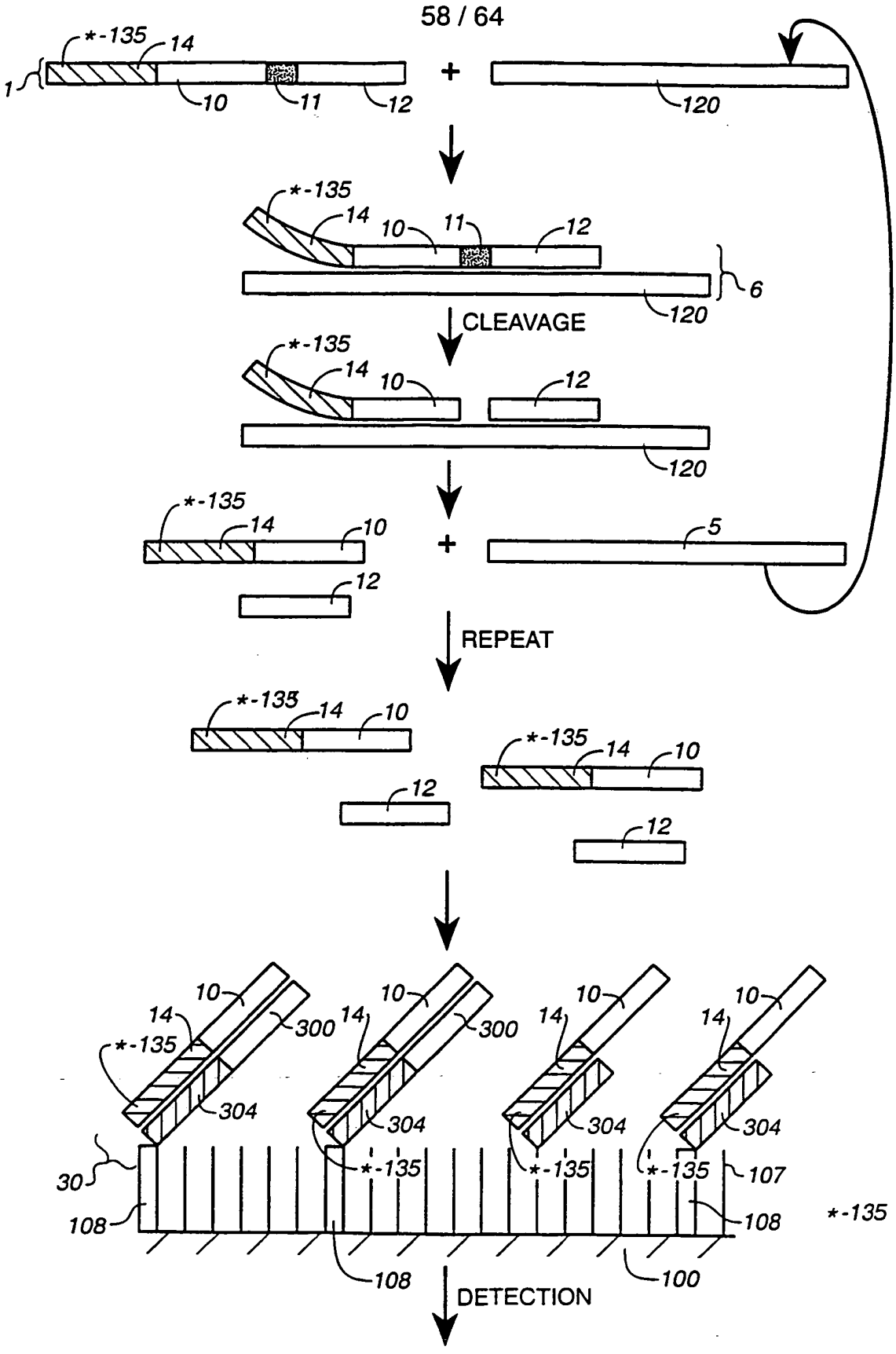


FIG._31

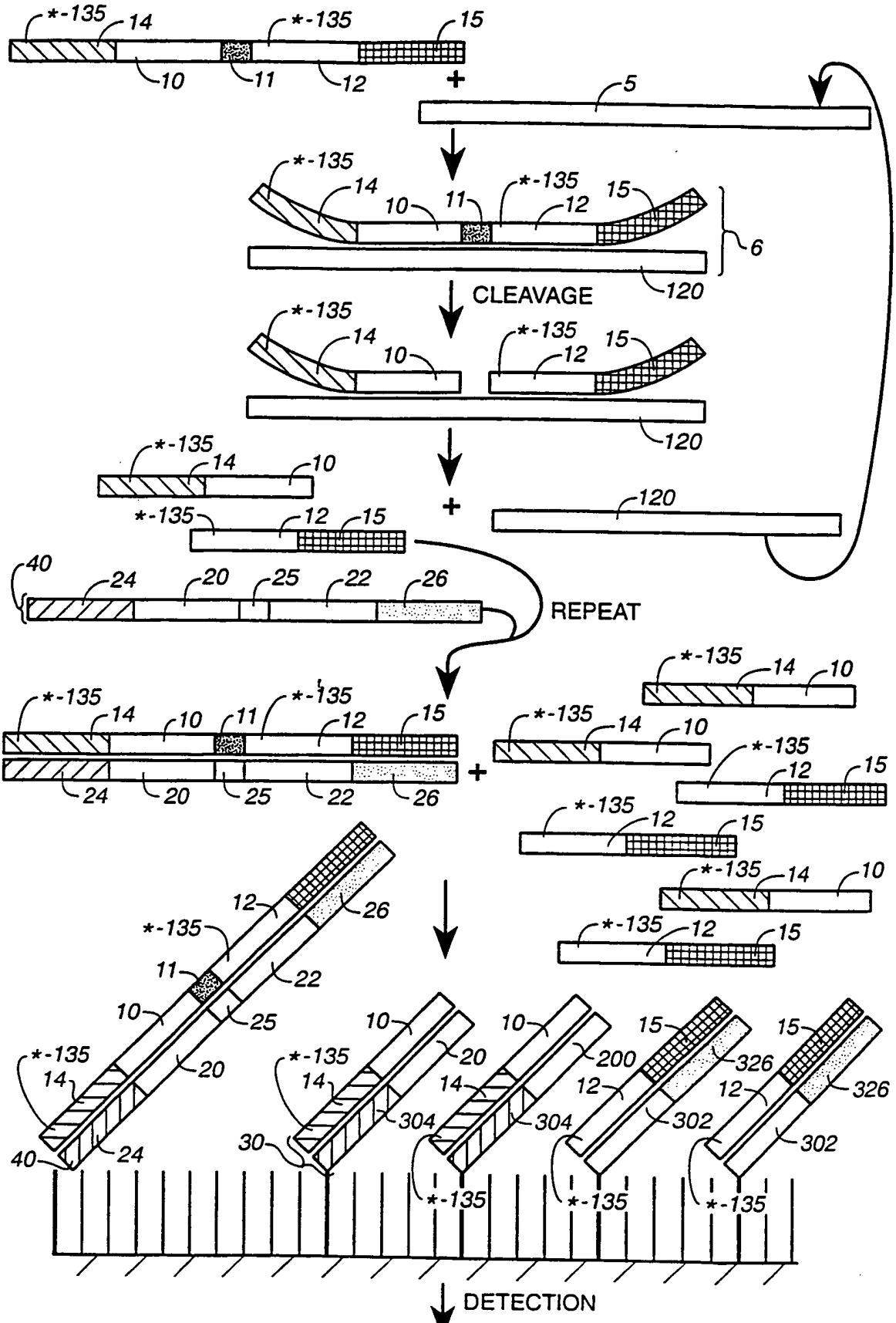


FIG._32

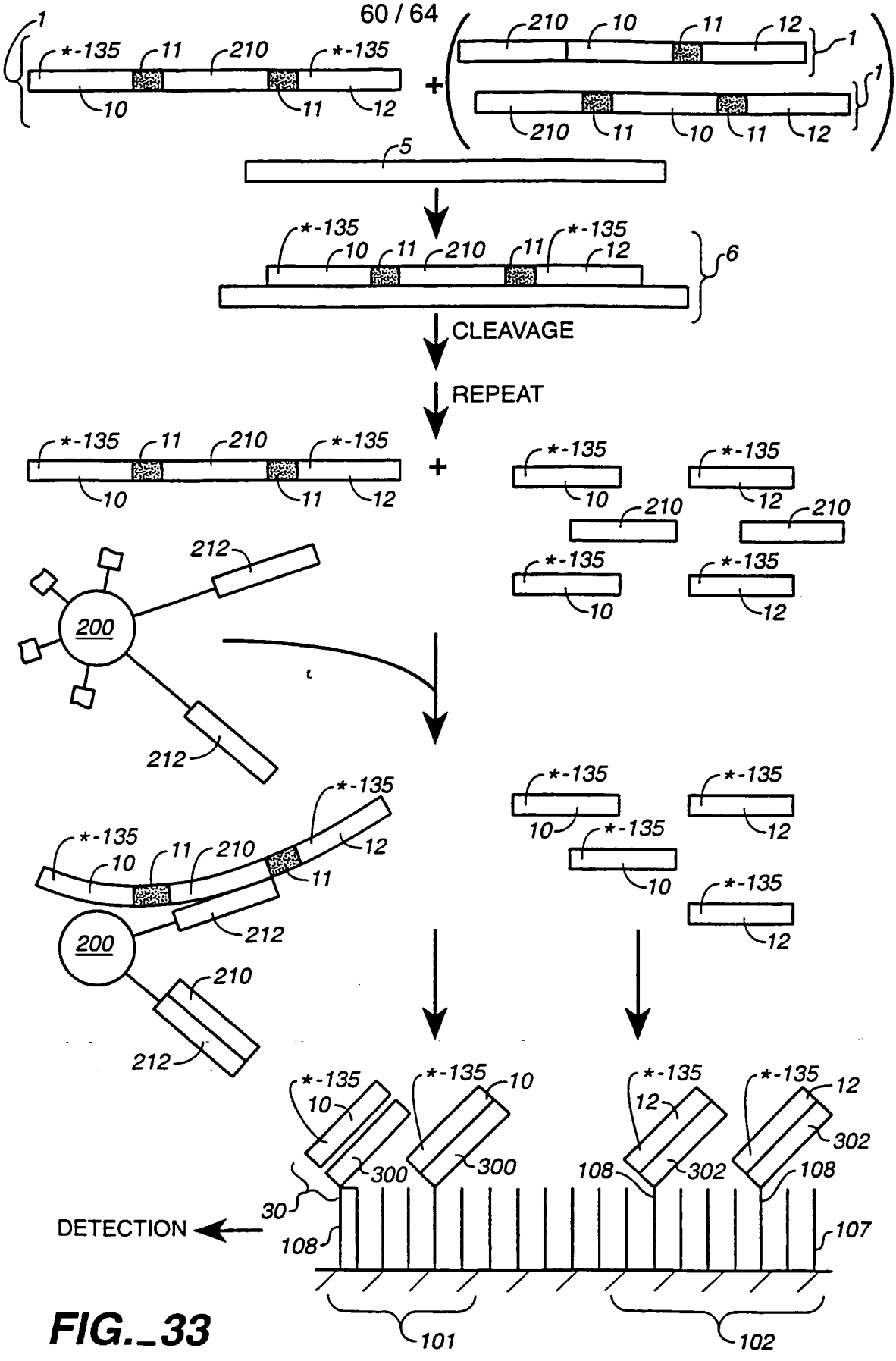


FIG._33

FIG._34

FIG._34A

FIG._34B

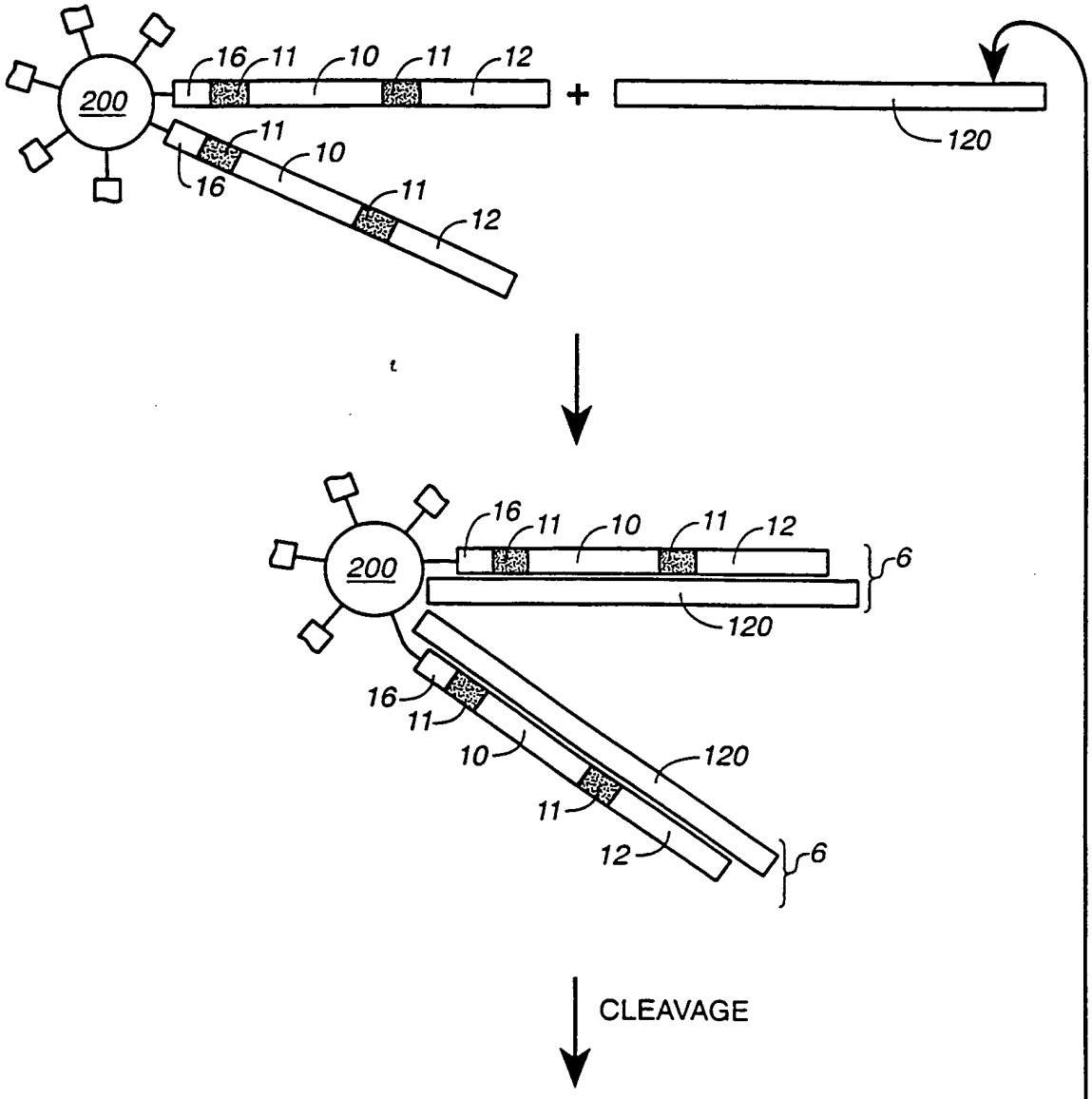


FIG._34A

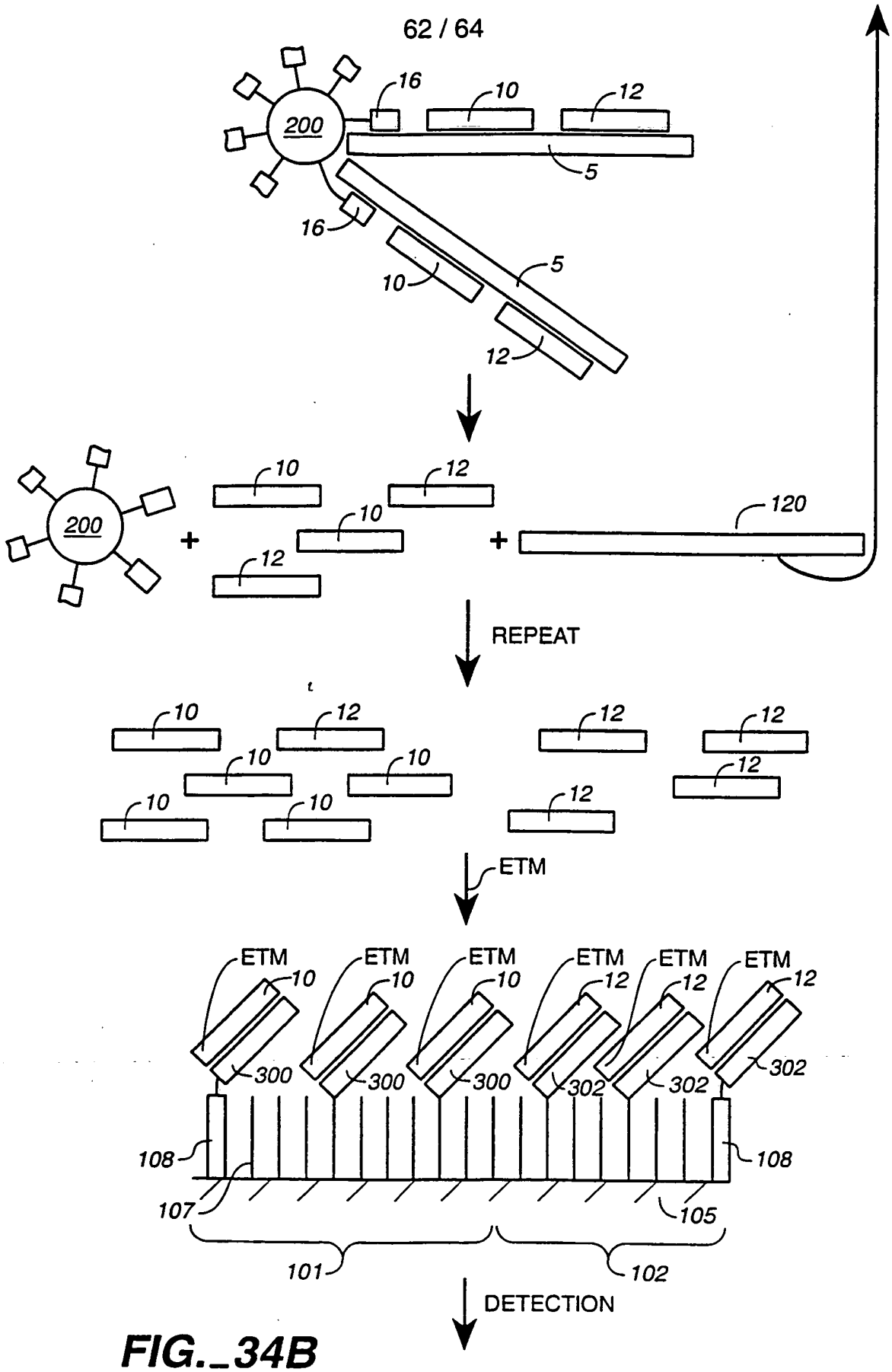


FIG._34B

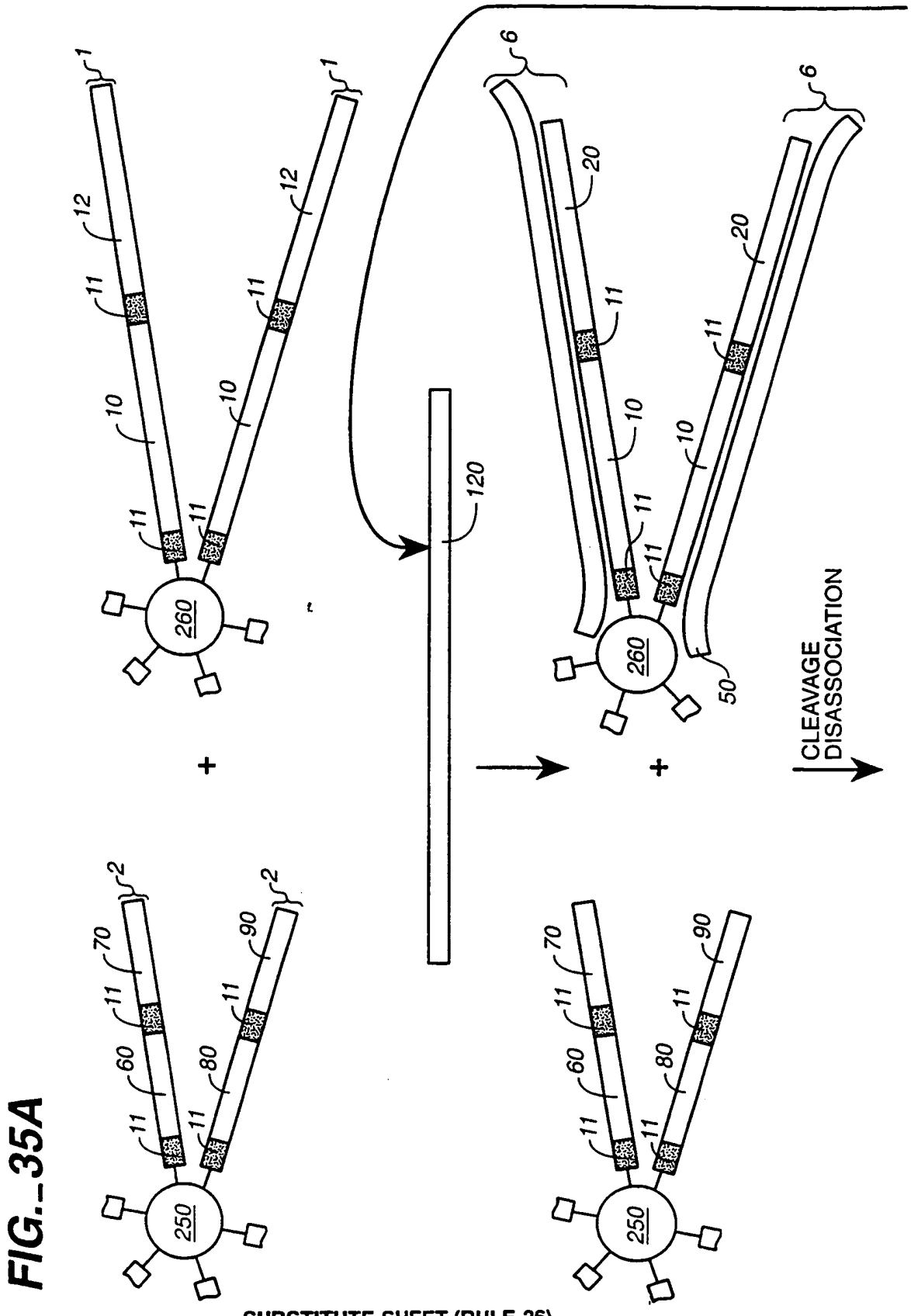
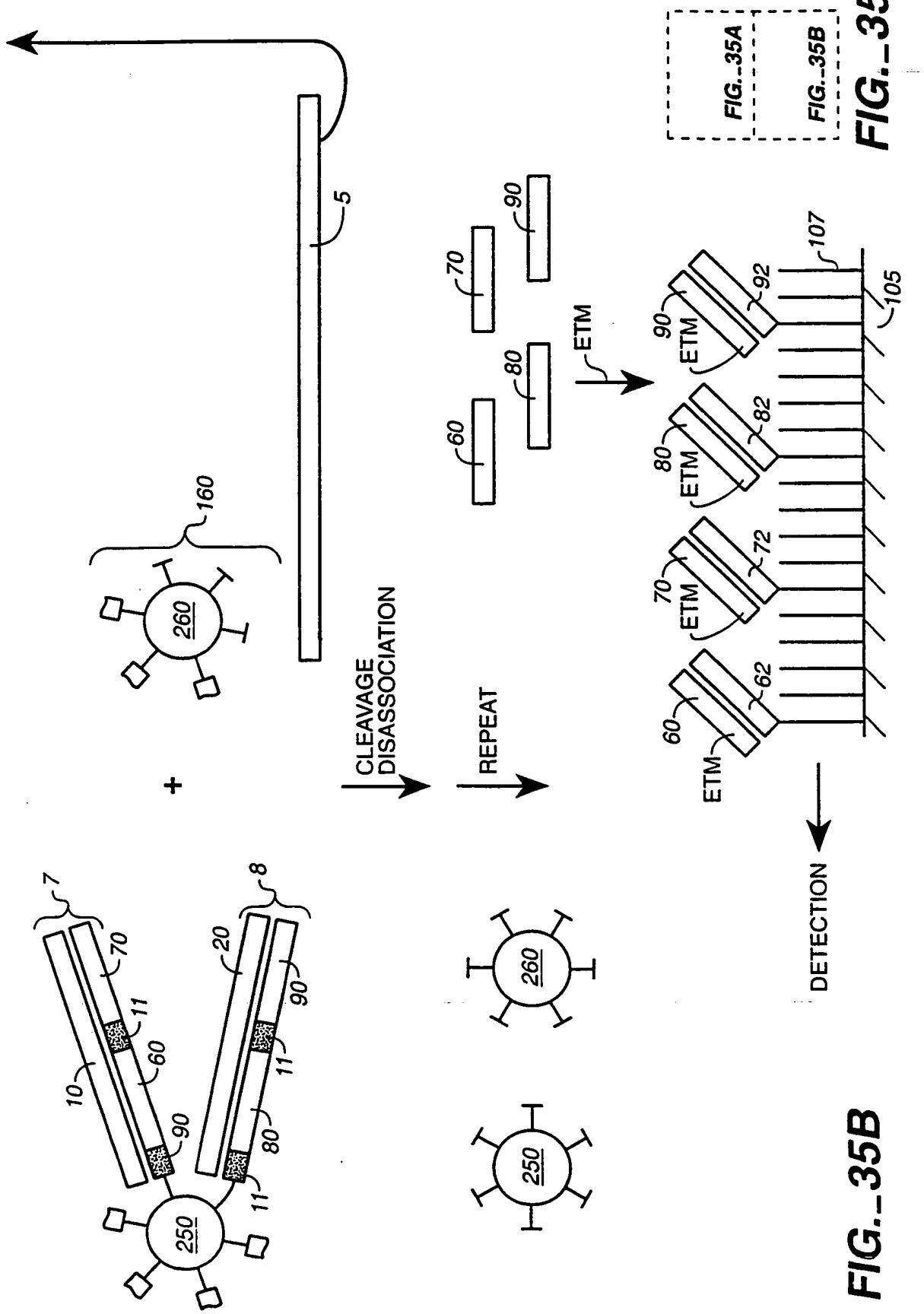


FIG._35A



PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68 | A3

(11) International Publication Number: **WO 99/37819**

(43) International Publication Date: 29 July 1999 (29.07.99)

(21) International Application Number: PCT/US99/01705

(22) International Filing Date: 27 January 1999 (27.01.99)

(30) Priority Data:

09/014,304	27 January 1998 (27.01.98)	US
60/073,011	29 January 1998 (29.01.98)	US
60/078,102	16 March 1998 (16.03.98)	US
60/084,425	6 May 1998 (06.05.98)	US
60/084,509	6 May 1998 (06.05.98)	US
09/135,183	17 August 1998 (17.08.98)	US

(71) Applicant *(for all designated States except US)*: CLINICAL MICRO SENSORS, INC. [US/US]; 101 Waverly Avenue, Pasadena, CA 91105 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: KAYYEM, Jon, Faiz [US/US]; 428 South Sierra Bonita Avenue, Pasadena, CA 91106 (US).

(74) Agents: SILVA, Robin, M. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111–4187 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report: 14 October 1999 (14.10.99)

(54) Title: AMPLIFICATION OF NUCLEIC ACIDS WITH ELECTRONIC DETECTION

(57) Abstract

The invention relates to compositions and methods useful in the detection of nucleic acids using a variety of amplification techniques, including both signal amplification and target amplification. Detection proceeds through the use of an electron transfer moiety (ETM) that is associated with the nucleic acid, either directly or indirectly, to allow electronic detection of the ETM using an electrode.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/US 99/01705

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 40712 A (CALIFORNIA INST OF TECHN) 19 December 1996 (1996-12-19) see whole doc. esp. claims and examples	1,13-16
A	UTO Y ET AL: "Electrochemical analysis of DNA amplified by the polymerase chain reaction with a ferrocenylated oligonucleotide" ANALYTICAL BIOCHEMISTRY, vol. 250, no. 250, 1997, pages 122-124 124, XP002106964 ISSN: 0003-2697	
A	WO 97 09337 A (DEUTSCHES KREBSFORSCH ;SCHUETTE DAGMAR (DE); WIESSLER MANFRED (DE)) 13 March 1997 (1997-03-13) the whole document	

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 August 1999	27/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/01705

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 20162 A (GOZIN MICHAEL ;YU CHANGJUN (US); KAYYEM JON F (US); CLINICAL MICRO) 14 May 1998 (1998-05-14) cited in the application the whole document	13-20
P,X	WO 98 57159 A (CLINICAL MICRO SENSORS INC) 17 December 1998 (1998-12-17) see esp. claims	13-16

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

additional components of the conductive oligomer. Thus, for example, when Structure 13 oligomers are used, a subunit comprises at least the first Y group.

A preferred method comprises 1) adding an ethyl pyridine or trimethylsilylethyl protecting group to a sulfur atom attached to a first subunit of a conductive oligomer, generally done by adding a vinyl pyridine or trimethylsilylethyl group to a sulfhydryl; 2) adding additional subunits to form the conductive oligomer; 3) adding at least a first nucleoside to the conductive oligomer; 4) adding additional nucleosides to the first nucleoside to form a nucleic acid; 5) attaching the conductive oligomer to the gold electrode. This may also be done in the absence of nucleosides, as is described in the Examples.

The above method may also be used to attach insulator molecules to a gold electrode.

In a preferred embodiment, a monolayer comprising conductive oligomers (and optionally insulators) is added to the electrode. Generally, the chemistry of addition is similar to or the same as the addition of conductive oligomers to the electrode, i.e. using a sulfur atom for attachment to a gold electrode, etc. Compositions comprising monolayers in addition to the conductive oligomers covalently attached to nucleic acids may be made in at least one of five ways: (1) addition of the monolayer, followed by subsequent addition of the attachment linker-nucleic acid complex; (2) addition of theattachment linker-nucleic acid complex followed by addition of the monolayer; (3) simultaneous addition of the monolayer and attachment linker-nucleic acid complex; (4) formation of a monolayer (using any of 1, 2 or 3) which includes attachment linkers which terminate in a functional moiety suitable for attachment of a completed nucleic acid; or (5) formation of a monolayer which includes attachment linkers which terminate in a functional moiety suitable for nucleic acid synthesis, i.e. the nucleic acid is synthesized on the surface of the monolayer as is known in the art. Such suitable functional moieties include, but are not limited to, nucleosides, amino groups, carboxyl groups, protected sulfur moieties, or hydroxyl groups for phosphoramidite additions. The examples describe the formation of a monolayer on a gold electrode using the preferred method (1).

In a preferred embodiment, the nucleic acid is a peptide nucleic acid or analog. In this embodiment, the invention provides peptide nucleic acids with at least one covalently attached ETM or attachment linker. In a preferred embodiment, these moieties are covalently attached to an monomeric subunit of the PNA. By "monomeric subunit of PNA" herein is meant the -NH-CH_2CH_2-N($COCH_2$-Base)-CH_2-CO- monomer, or derivatives (herein included within the definition of "nucleoside") of PNA. For example, the number of carbon atoms in the PNA backbone may be altered; see generally Nielsen et al., Chem. Soc. Rev. 1997 page 73, which discloses a number of PNA derivatives, herein expressly incorporated by reference. Similarly, the amide bond linking the base to the backbone may be altered;

phosphoramide and sulfuramide bonds may be used. Alternatively, the moieties are attached to an internal monomeric subunit. By "internal" herein is meant that the monomeric subunit is not either the N-terminal monomeric subunit or the C-terminal monomeric subunit. In this embodiment, the moieties can be attached either to a base or to the backbone of the monomeric subunit. Attachment to the base is done as outlined herein or known in the literature. In general, the moieties are added to a base which is then incorporated into a PNA as outlined herein. The base may be either protected, as required for incorporation into the PNA synthetic reaction, or derivatized, to allow incorporation, either prior to the addition of the chemical substituent or afterwards. Protection and derivatization of the bases is shown in Figures 24-27 of WO98/20162. The bases can then be incorporated into monomeric subunits as shown in Figure 28 of WO98/20162. Figures 29 and 30 of WO98/20162 depict two different chemical substituents, an ETM and a conductive oligomer, attached at a base. Figure 29 depicts a representative synthesis of a PNA monomeric subunit with a ferrocene attached to a uracil base. Figure 30 depicts the synthesis of a three unit conductive oligomer attached to a uracil base.

In a preferred embodiment, the moieties are covalently attached to the backbone of the PNA monomer. The attachment is generally to one of the unsubstituted carbon atoms of the monomeric subunit, preferably the α-carbon of the backbone, although attachment at either of the carbon 1 or 2 positions, or the α-carbon of the amide bond linking the base to the backbone may be done. In the case of PNA analogs, other carbons or atoms may be substituted as well. In a preferred embodiment, moieties are added at the α-carbon atoms, either to a terminal monomeric subunit or an internal one.

In this embodiment, a modified monomeric subunit is synthesized with an ETM or an attachment linker, or a functional group for its attachment, and then the base is added and the modified monomer can be incorporated into a growing PNA chain. Figure 31 of WO98/20162 depicts the synthesis of a conductive oligomer covalently attached to the backbone of a PNA monomeric subunit, and Figure 32 of WO98/20162 depicts the synthesis of a ferrocene attached to the backbone of a monomeric subunit.

Once generated, the monomeric subunits with covalently attached moieties are incorporated into a PNA using the techniques outlined in Will et al., Tetrahedron 51(44):12069-12082 (1995), and Vanderlaan et al., Tett. Let. 38:2249-2252 (1997), both of which are hereby expressly incorporated in their entirety. These procedures allow the addition of chemical substituents to peptide nucleic acids without destroying the chemical substituents.

As will be appreciated by those in the art, electrodes may be made that have any combination of nucleic acids, conductive oligomers and insulators.

The compositions of the invention may additionally contain one or more labels at any position. By "label" herein is meant an element (e.g. an isotope) or chemical compound that is attached to enable the detection of the compound. Preferred labels are radioactive isotopic labels, and colored or fluorescent dyes. The labels may be incorporated into the compound at any position. In addition, the compositions of the invention may also contain other moieties such as cross-linking agents to facilitate cross-linking of the target-probe complex. See for example, Lukhtanov et al., Nucl. Acids. Res. 24(4):683 (1996) and Tabone et al., Biochem. 33:375 (1994), both of which are expressly incorporated by reference.

When mechanism-1 systems are used, detection probes are covalently attached to the electrode, as above for capture probes. The detection probes are either substantially complementary to a portion of the target sequence (direct detection), or to a portion of a label probe (sandwich assay), as is depicted in the Figures.

As for all of the methods outlined herein, it may be necessary to either remove unreacted primers or configure the detection systems such that unreacted primers are not detected, depending on the method used. For example, for all of the systems, the removal of unreacted primers based on size differences can be done, or in some cases, by binding to a solid support such as a bead, using a separation tag. In addition, for PCR, SDA and NASBA, detection specificity will utilize portions of the non-primer newly synthesized strands, such that unextended primers will not be bound by capture probes on an electrode, for example. Alternatively, for example, in CPT, the first probe sequence may comprise a separation tag (e.g. biotin) or sequence (e.g. a unique sequence), that allow the binding of the unreacted primers and the cleaved first probe sequences; the use of labels in the second probe sequence (for direct detection) or the use of the second probe sequence for the basis of the capture onto an electrode or binding to a detection probe ensures that unreacted probes are not detected. Similarly, in LCR, the use of one primer for capture and the other for either label incorporation (direct detection) or detection specificity allows that detection will only proceed for the modified primers.

Once made, the compositions find use in a number of applications, as described herein. In particular, the compositions of the invention find use in hybridization assays. As will be appreciated by those in the art, electrodes can be made that have a single species of nucleic acid, i.e. a single nucleic acid sequence, or multiple nucleic acid species.

In addition, as outlined herein, the use of a solid support such as an electrode enables the use of these gene probes in an array form. The use of oligonucleotide arrays are well known in the art. In addition, techniques are known for "addressing" locations within an electrode and for the surface modification of electrodes. Thus, in a preferred embodiment, arrays of different nucleic acids are laid

down on the electrode, each of which are covalently attached to the electrode via a conductive linker. In this embodiment, the number of different probe species of oligonucleotides may vary widely, from one to thousands, with from about 4 to about 100,000 being preferred, and from about 10 to about 10,000 being particularly preferred.

Once the assay complexes of the invention are made, that minimally comprise a target sequence and an ETM, detection proceeds with electronic initiation. Without being limited by the mechanism or theory, detection is based on the transfer of electrons from the ETM to the electrode.

Detection of electron transfer, i.e. the presence of the ETMs, is generally initiated electronically, with voltage being preferred. A potential is applied to the assay complex. Precise control and variations in the applied potential can be via a potentiostat and either a three electrode system (one reference, one sample (or working) and one counter electrode) or a two electrode system (one sample and one counter electrode). This allows matching of applied potential to peak potential of the system which depends in part on the choice of ETMs and in part on the conductive oligomer used, the composition and integrity of the monolayer, and what type of reference electrode is used. As described herein, ferrocene is a preferred ETM.

In a preferred embodiment, a co-reductant or co-oxidant (collectively, co-redoxant) is used, as an additional electron source or sink. See generally Sato et al., Bull. Chem. Soc. Jpn 66:1032 (1993); Uosaki et al., Electrochimica Acta 36:1799 (1991); and Alleman et al., J. Phys. Chem 100:17050 (1996); all of which are incorporated by reference.

In a preferred embodiment, an input electron source in solution is used in the initiation of electron transfer, preferably when initiation and detection are being done using DC current or at AC frequencies where diffusion is not limiting. In general, as will be appreciated by those in the art, preferred embodiments utilize monolayers that contain a minimum of "holes", such that short-circuiting of the system is avoided. This may be done in several general ways. In a preferred embodiment, an input electron source is used that has a lower or similar redox potential than the ETM of the label probe. Thus, at voltages above the redox potential of the input electron source, both the ETM and the input electron source are oxidized and can thus donate electrons; the ETM donates an electron to the electrode and the input source donates to the ETM. For example, ferrocene, as a ETM attached to the compositions of the invention as described in the examples, has a redox potential of roughly 200 mV in aqueous solution (which can change significantly depending on what the ferrocene is bound to, the manner of the linkage and the presence of any substitution groups). Ferrocyanide, an electron source, has a redox potential of roughly 200 mV as well (in aqueous solution). Accordingly, at or above voltages of roughly 200 mV, ferrocene is converted to ferricenium, which then transfers an

electron to the electrode. Now the ferricyanide can be oxidized to transfer an electron to the ETM. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the ETM attached to the nucleic acid. The rate of electron donation or acceptance will be limited by the rate of diffusion of the co-reductant, the electron transfer between the co-reductant and the ETM, which in turn is affected by the concentration and size, etc.

Alternatively, input electron sources that have lower redox potentials than the ETM are used. At voltages less than the redox potential of the ETM, but higher than the redox potential of the electron source, the input source such as ferrocyanide is unable to be oxided and thus is unable to donate an electron to the ETM; i.e. no electron transfer occurs. Once ferrocene is oxidized, then there is a pathway for electron transfer.

In an alternate preferred embodiment, an input electron source is used that has a higher redox potential than the ETM of the label probe. For example, luminol, an electron source, has a redox potential of roughly 720 mV. At voltages higher than the redox potential of the ETM, but lower than the redox potential of the electron source, i.e. 200 - 720 mV, the ferrocene is oxided, and transfers a single electron to the electrode via the conductive oligomer. However, the ETM is unable to accept any electrons from the luminol electron source, since the voltages are less than the redox potential of the luminol. However, at or above the redox potential of luminol, the luminol then transfers an electron to the ETM, allowing rapid and repeated electron transfer. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the ETM of the label probe.

Luminol has the added benefit of becoming a chemiluminiscent species upon oxidation (see Jirka et al., Analytica Chimica Acta 284:345 (1993)), thus allowing photo-detection of electron transfer from the ETM to the electrode. Thus, as long as the luminol is unable to contact the electrode directly, i.e. in the presence of the SAM such that there is no efficient electron transfer pathway to the electrode, luminol can only be oxidized by transferring an electron to the ETM on the label probe. When the ETM is not present, i.e. when the target sequence is not hybridized to the composition of the invention, luminol is not significantly oxidized, resulting in a low photon emission and thus a low (if any) signal from the luminol. In the presence of the target, a much larger signal is generated. Thus, the measure of luminol oxidation by photon emission is an indirect measurement of the ability of the ETM to donate electrons to the electrode. Furthermore, since photon detection is generally more sensitive than electronic detection, the sensitivity of the system may be increased. Initial results suggest that luminescence may depend on hydrogen peroxide concentration, pH, and luminol concentration, the latter of which appears to be non-linear.

Suitable electron source molecules are well known in the art, and include, but are not limited to, ferricyanide, and luminol.

Alternatively, output electron acceptors or sinks could be used, i.e. the above reactions could be run in reverse, with the ETM such as a metallocene receiving an electron from the electrode, converting it to the metallicenium, with the output electron acceptor then accepting the electron rapidly and repeatedly. In this embodiment, cobalticenium is the preferred ETM.

The presence of the ETMs at the surface of the monolayer can be detected in a variety of ways. A variety of detection methods may be used, including, but not limited to, optical detection (as a result of spectral changes upon changes in redox states), which includes fluorescence, phosphorescence, luminiscence, chemiluminescence, electrochemiluminescence, and refractive index; and electronic detection, including, but not limited to, amperommetry, voltammetry, capacitance and impedence. These methods include time or frequency dependent methods based on AC or DC currents, pulsed methods, lock-in techniques, filtering (high pass, low pass, band pass), and time-resolved techniques including time-resolved fluoroscence.

In one embodiment, the efficient transfer of electrons from the ETM to the electrode results in stereotyped changes in the redox state of the ETM. With many ETMs including the complexes of ruthenium containing bipyridine, pyridine and imidazole rings, these changes in redox state are associated with changes in spectral properties. Significant differences in absorbance are observed between reduced and oxidized states for these molecules. See for example Fabbrizzi et al., Chem. Soc. Rev. 1995 pp197-202). These differences can be monitored using a spectrophotometer or simple photomultiplier tube device.

In this embodiment, possible electron donors and acceptors include all the derivatives listed above for photoactivation or initiation. Preferred electron donors and acceptors have characteristically large spectral changes upon oxidation and reduction resulting in highly sensitive monitoring of electron transfer. Such examples include $Ru(NH_3)_4py$ and $Ru(bpy)_2im$ as preferred examples. It should be understood that only the donor or acceptor that is being monitored by absorbance need have ideal spectral characteristics.

In a preferred embodiment, the electron transfer is detected fluorometrically. Numerous transition metal complexes, including those of ruthenium, have distinct fluorescence properties. Therefore, the change in redox state of the electron donors and electron acceptors attached to the nucleic acid can be monitored very sensitively using fluorescence, for example with $Ru(4,7\text{-biphenyl}_2\text{-phenanthroline})_3^{2+}$. The production of this compound can be easily measured using standard fluorescence assay

techniques. For example, laser induced fluorescence can be recorded in a standard single cell fluorimeter, a flow through "on-line" fluorimeter (such as those attached to a chromatography system) or a multi-sample "plate-reader" similar to those marketed for 96-well immuno assays.

Alternatively, fluorescence can be measured using fiber optic sensors with nucleic acid probes in solution or attached to the fiber optic. Fluorescence is monitored using a photomultiplier tube or other light detection instrument attached to the fiber optic. The advantage of this system is the extremely small volumes of sample that can be assayed.

In addition, scanning fluorescence detectors such as the FluorImager sold by Molecular Dynamics are ideally suited to monitoring the fluorescence of modified nucleic acid molecules arrayed on solid surfaces. The advantage of this system is the large number of electron transfer probes that can be scanned at once using chips covered with thousands of distinct nucleic acid probes.

Many transition metal complexes display fluorescence with large Stokes shifts. Suitable examples include bis- and trisphenanthroline complexes and bis- and trisbipyridyl complexes of transition metals such as ruthenium (see Juris, A., Balzani, V., et. al. Coord. Chem. Rev., V. 84, p. 85-277, 1988). Preferred examples display efficient fluorescence (reasonably high quantum yields) as well as low reorganization energies. These include Ru(4,7-biphenyl$_2$-phenanthroline)$_3^{2+}$, Ru(4,4′-diphenyl-2,2′-bipyridine)$_3^{2+}$ and platinum complexes (see Cummings et al., J. Am. Chem. Soc. 118:1949-1960 (1996), incorporated by reference). Alternatively, a *reduction* in fluorescence associated with hybridization can be measured using these systems.

In a further embodiment, electrochemiluminescence is used as the basis of the electron transfer detection. With some ETMs such as $Ru^{2+}(bpy)_3$, direct luminescence accompanies excited state decay. Changes in this property are associated with nucleic acid hybridization and can be monitored with a simple photomultiplier tube arrangement (see Blackburn, G. F. *Clin. Chem.* **37**: 1534-1539 (1991); and Juris et al., supra.

In a preferred embodiment, electronic detection is used, including amperommetry, voltammetry, capacitance; and impedence. Suitable techniques include, but are not limited to, electrogravimetry; coulometry (including controlled potential coulometry and constant current coulometry); voltametry (cyclic voltametry, pulse voltametry (normal pulse voltametry, square wave voltametry, differential pulse voltametry, Osteryoung square wave voltametry, and coulostatic pulse techniques); stripping analysis (aniodic stripping analysis, cathiodic stripping analysis, square wave stripping voltammetry); conductance measurements (electrolytic conductance, direct analysis); time-dependent electrochemical analyses (chronoamperometry, chronopotentiometry, cyclic chronopotentiometry and

amperometry, AC polography, chronogalvametry, and chronocoulometry); AC impedance measurement; capacitance measurement; AC voltametry; and photoelectrochemistry.

In a preferred embodiment, monitoring electron transfer is via amperometric detection. This method of detection involves applying a potential (as compared to a separate reference electrode) between the nucleic acid-conjugated electrode and a reference (counter) electrode in the sample containing target genes of interest. Electron transfer of differing efficiencies is induced in samples in the presence or absence of target nucleic acid; that is, the presence or absence of the target nucleic acid, and thus the label probe, can result in different currents.

The device for measuring electron transfer amperometrically involves sensitive current detection and includes a means of controlling the voltage potential, usually a potentiostat. This voltage is optimized with reference to the potential of the electron donating complex on the label probe. Possible electron donating complexes include those previously mentioned with complexes of iron, osmium, platinum, cobalt, rhenium and ruthenium being preferred and complexes of iron being most preferred.

In a preferred embodiment, alternative electron detection modes are utilized. For example, potentiometric (or voltammetric) measurements involve non-faradaic (no net current flow) processes and are utilized traditionally in pH and other ion detectors. Similar sensors are used to monitor electron transfer between the ETM and the electrode. In addition, other properties of insulators (such as resistance) and of conductors (such as conductivity, impedance and capicitance) could be used to monitor electron transfer between ETM and the electrode. Finally, any system that generates a current (such as electron transfer) also generates a small magnetic field, which may be monitored in some embodiments.

It should be understood that one benefit of the fast rates of electron transfer observed in the compositions of the invention is that time resolution can greatly enhance the signal-to-noise results of monitors based on absorbance, fluorescence and electronic current. The fast rates of electron transfer of the present invention result both in high signals and stereotyped delays between electron transfer initiation and completion. By amplifying signals of particular delays, such as through the use of pulsed initiation of electron transfer and "lock-in" amplifiers of detection, and Fourier transforms.

In a preferred embodiment, electron transfer is initiated using alternating current (AC) methods. Without being bound by theory, it appears that ETMs, bound to an electrode, generally respond similarly to an AC voltage across a circuit containing resistors and capacitors. Basically, any methods which enable the determination of the nature of these complexes, which act as a resistor and capacitor, can be used as the basis of detection. Surprisingly, traditional electrochemical theory, such

as exemplified in Laviron et al., J. Electroanal. Chem. 97:135 (1979) and Laviron et al., J. Electroanal. Chem. 105:35 (1979), both of which are incorporated by reference, do not accurately model the systems described herein, except for very small E_{AC} (less than 10 mV) and relatively large numbers of molecules. That is, the AC current (I) is not accurately described by Laviron's equation. This may be due in part to the fact that this theory assumes an unlimited source and sink of electrons, which is not true in the present systems.

Accordingly, alternate equations were developed, using the Nernst equation and first principles to develop a model which more closely simulates the results. This was derived as follows. The Nernst equation, Equation 1 below, describes the ratio of oxidized (O) to reduced (R) molecules (number of molecules = n) at any given voltage and temperature, since not every molecule gets oxidized at the same oxidation potential.

Equation 1

$$E_{DC}=E_0+\frac{RT}{nF}\ \ell n\frac{[O]}{[R]} \qquad (1)$$

E_{DC} is the electrode potential, E_0 is the formal potential of the metal complex, R is the gas constant, T is the temperature in degrees Kelvin, n is the number of electrons transferred, F is faraday's constant, [O] is the concentration of oxidized molecules and [R] is the concentration of reduced molecules.

The Nernst equation can be rearranged as shown in Equations 2 and 3:

Equation 2

$$E_{DC}-E_0=\frac{RT}{nF}\ \ell n\frac{[O]}{[R]} \qquad (2)$$

E_{DC} is the DC component of the potential.

Equation 3

$$\exp^{\frac{nF}{RT}(E_{DC}\ -\ E_0)}=\frac{[O]}{[R]} \qquad (3)$$

Equation 3 can be rearranged as follows, using normalization of the concentration to equal 1 for simplicity, as shown in Equations 4, 5 and 6. This requires the subsequent multiplication by the total number of molecules.

Equation 4 $[O] + [R] = 1$

Equation 5 $[O] = 1 - [R]$

Equation 6 $[R] = 1 - [O]$

Plugging Equation 5 and 6 into Equation 3, and the fact that nF/RT equals 38.9 V^{-1}, for n=1, gives Equations 7 and 8, which define [O] and [R], respectively:

Equation 7

$$[O] = \frac{\exp^{38.9(E-E_0)}}{1 + \exp^{38.9(E-E_0)}} \qquad (4)$$

Equation 8

$$[R] = \frac{1}{1 + \exp^{38.9\,(E\,-\,E_0)}} \qquad (5)$$

Taking into consideration the generation of an AC faradaic current, the ratio of [O]/[R] at any given potential must be evaluated. At a particular E_{DC} with an applied E_{AC}, as is generally described herein, at the apex of the E_{AC} more molecules will be in the oxidized state, since the voltage on the surface is now (E_{DC} + E_{AC}); at the bottom, more will be reduced since the voltage is lower. Therefore, the AC current at a given E_{DC} will be dictated by both the AC and DC voltages, as well as the shape of the Nernstian curve. Specifically, if the number of oxidized molecules at the bottom of the AC cycle is subtracted from the amount at the top of the AC cycle, the total change in a given AC cycle is obtained, as is generally described by Equation 9. Dividing by 2 then gives the AC amplitude.

Equation 9

$$i_{AC} = \frac{(\text{electrons at } [E_{DC} + E_{AC}]) - (\text{electrons at } [E_{DC} - E_{AC}])}{2}$$

Equation 10 thus describes the AC current which should result:

Equation 10

$$i_{AC} = C_0\, F\omega\, \tfrac{1}{2}\, ([O]_{E_{DC}\,+\,E_{AC}} - [O]_{E_{DC}\,-\,E_{AC}}) \qquad (6)$$

As depicted in Equation 11, the total AC current will be the number of redox molecules C), times faraday's constant (F), times the AC frequency (ω), times 0.5 (to take into account the AC amplitude), times the ratios derived above in Equation 7. The AC voltage is approximated by the average, $E_{AC}2/\pi$.

Equation 11

$$i_C = \frac{C_0 F\omega}{2}\left(\frac{\exp^{38.9\,[E_{DC} + \frac{2E_{AC}}{\pi} - E_0]}}{1 + \exp^{38.9\,[E_{DC} + \frac{2E_{AC}}{\pi} - E_0]}}\right) - \frac{\exp^{38.9\,[E_{DC} - \frac{2E_{AC}}{\pi} - E_0]}}{1 + \exp^{38.9\,[E_{DC} - \frac{2E_{AC}}{\pi} - E}} \quad (7)$$

Using Equation 11, simulations were generated using increasing overpotential (AC voltage). Figure 22A of WO98/20162 shows one of these simulations, while Figure 22B depicts a simulation based on traditional theory. Figures 23A and 23B depicts actual experimental data using the Fc-wire of Example 7 of WO98/20162 plotted with the simulation, and shows that the model fits the experimental data very well. In some cases the current is smaller than predicted, however this has been shown to be caused by ferrocene degradation which may be remedied in a number of ways. However, Equation 11 does not incorporate the effect of electron transfer rate nor of instrument factors. Electron transfer rate is important when the rate is close to or lower than the applied frequency. Thus, the true i_{AC} should be a function of all three, as depicted in Equation 12.

Equation 12

$$i_{AC} = f(\text{Nernst factors})f(k_{ET})f(\text{instrument factors})$$

These equations can be used to model and predict the expected AC currents in systems which use input signals comprising both AC and DC components. As outlined above, traditional theory surprisingly does not model these systems at all, except for very low voltages.

In general, non-specifically bound label probes/ETMs show differences in impedance (i.e. higher impedances) than when the label probes containing the ETMs are specifically bound in the correct orientation. In a preferred embodiment, the non-specifically bound material is washed away, resulting in an effective impedance of infinity. Thus, AC detection gives several advantages as is generally discussed below, including an increase in sensitivity, and the ability to "filter out" background noise. In particular, changes in impedance (including, for example, bulk impedance) as between non-specific binding of ETM-containing probes and target-specific assay complex formation may be monitored.

Accordingly, when using AC initiation and detection methods, the frequency response of the system changes as a result of the presence of the ETM. By "frequency response" herein is meant a modification of signals as a result of electron transfer between the electrode and the ETM. This modification is different depending on signal frequency. A frequency response includes AC currents at one or more frequencies, phase shifts, DC offset voltages, faradaic impedance, etc.

Once the assay complex including the target sequence and the ETM is made, a first input electrical signal is then applied to the system, preferably via at least the sample electrode (containing the complexes of the invention) and the counter electrode, to initiate electron transfer between the electrode and the ETM. Three electrode systems may also be used, with the voltage applied to the reference and working electrodes. The first input signal comprises at least an AC component. The AC component may be of variable amplitude and frequency. Generally, for use in the present methods, the AC amplitude ranges from about 1 mV to about 1.1 V, with from about 10 mV to about 800 mV being preferred, and from about 10 mV to about 500 mV being especially preferred. The AC frequency ranges from about 0.01 Hz to about 100 MHz, with from about 10 Hz to about 10 MHz being preferred, and from about 100 Hz to about 20 MHz being especially preferred.

The use of combinations of AC and DC signals gives a variety of advantages, including surprising sensitivity and signal maximization.

In a preferred embodiment, the first input signal comprises a DC component and an AC component. That is, a DC offset voltage between the sample and counter electrodes is swept through the electrochemical potential of the ETM (for example, when ferrocene is used, the sweep is generally from 0 to 500 mV) (or alternatively, the working electrode is grounded and the reference electrode is swept from 0 to -500 mV). The sweep is used to identify the DC voltage at which the maximum response of the system is seen. This is generally at or about the electrochemical potential of the ETM. Once this voltage is determined, either a sweep or one or more uniform DC offset voltages may be used. DC offset voltages of from about -1 V to about +1.1 V are preferred, with from about -500 mV to about +800 mV being especially preferred, and from about -300 mV to about 500 mV being particularly preferred. In a preferred embodiment, the DC offset voltage is not zero. On top of the DC offset voltage, an AC signal component of variable amplitude and frequency is applied. If the ETM is present, and can respond to the AC perturbation, an AC current will be produced due to electron transfer between the electrode and the ETM.

For defined systems, it may be sufficient to apply a single input signal to differentiate between the presence and absence of the ETM (i.e. the presence of the target sequence) nucleic acid. Alternatively, a plurality of input signals are applied. As outlined herein, this may take a variety of forms, including using multiple frequencies, multiple DC offset voltages, or multiple AC amplitudes, or combinations of any or all of these.

Thus, in a preferred embodiment, multiple DC offset voltages are used, although as outlined above, DC voltage sweeps are preferred. This may be done at a single frequency, or at two or more frequencies .

In a preferred embodiment, the AC amplitude is varied. Without being bound by theory, it appears that increasing the amplitude increases the driving force. Thus, higher amplitudes, which result in higher overpotentials give faster rates of electron transfer. Thus, generally, the same system gives an improved response (i.e. higher output signals) at any single frequency through the use of higher overpotentials at that frequency. Thus, the amplitude may be increased at high frequencies to increase the rate of electron transfer through the system, resulting in greater sensitivity. In addition, this may be used, for example, to induce responses in slower systems such as those that do not possess optimal spacing configurations.

In a preferred embodiment, measurements of the system are taken at at least two separate amplitudes or overpotentials, with measurements at a plurality of amplitudes being preferred. As noted above, changes in response as a result of changes in amplitude may form the basis of identification, calibration and quantification of the system. In addition, one or more AC frequencies can be used as well.

In a preferred embodiment, the AC frequency is varied. At different frequencies, different molecules respond in different ways. As will be appreciated by those in the art, increasing the frequency generally increases the output current. However, when the frequency is greater than the rate at which electrons may travel between the electrode and the ETM, higher frequencies result in a loss or decrease of output signal. At some point, the frequency will be greater than the rate of electron transfer between the ETM and the electrode, and then the output signal will also drop.

In one embodiment, detection utilizes a single measurement of output signal at a single frequency. That is, the frequency response of the system in the absence of target sequence, and thus the absence of label probe containing ETMs, can be previously determined to be very low at a particular high frequency. Using this information, any response at a particular frequency, will show the presence of the assay complex. That is, any response at a particular frequency is characteristic of the assay complex. Thus, it may only be necessary to use a single input high frequency, and any changes in frequency response is an indication that the ETM is present, and thus that the target sequence is present.

In addition, the use of AC techniques allows the significant reduction of background signals at any single frequency due to entities other than the ETMs, i.e. "locking out" or "filtering" unwanted signals. That is, the frequency response of a charge carrier or redox active molecule in solution will be limited by its diffusion coefficient and charge transfer coefficient. Accordingly, at high frequencies, a charge carrier may not diffuse rapidly enough to transfer its charge to the electrode, and/or the charge transfer kinetics may not be fast enough. This is particularly significant in embodiments that do not have good

monolayers, i.e. have partial or insufficient monolayers, i.e. where the solvent is accessible to the electrode. As outlined above, in DC techniques, the presence of "holes" where the electrode is accessible to the solvent can result in solvent charge carriers "short circuiting" the system, i.e. the reach the electrode and generate background signal. However, using the present AC techniques, one or more frequencies can be chosen that prevent a frequency response of one or more charge carriers in solution, whether or not a monolayer is present. This is particularly significant since many biological fluids such as blood contain significant amounts of redox active molecules which can interfere with amperometric detection methods.

In a preferred embodiment, measurements of the system are taken at at least two separate frequencies, with measurements at a plurality of frequencies being preferred. A plurality of frequencies includes a scan. For example, measuring the output signal, e.g., the AC current, at a low input frequency such as 1 - 20 Hz, and comparing the response to the output signal at high frequency such as 10 - 100 kHz will show a frequency response difference between the presence and absence of the ETM. In a preferred embodiment, the frequency response is determined at at least two, preferably at least about five, and more preferably at least about ten frequencies.

After transmitting the input signal to initiate electron transfer, an output signal is received or detected. The presence and magnitude of the output signal will depend on a number of factors, including the overpotential/amplitude of the input signal; the frequency of the input AC signal; the composition of the intervening medium; the DC offset; the environment of the system; the nature of the ETM; the solvent; and the type and concentration of salt. At a given input signal, the presence and magnitude of the output signal will depend in general on the presence or absence of the ETM, the placement and distance of the ETM from the surface of the monolayer and the character of the input signal. In some embodiments, it may be possible to distinguish between non-specific binding of label probes and the formation of target specific assay complexes containing label probes, on the basis of impedance.

In a preferred embodiment, the output signal comprises an AC current. As outlined above, the magnitude of the output current will depend on a number of parameters. By varying these parameters, the system may be optimized in a number of ways.

In general, AC currents generated in the present invention range from about 1 femptoamp to about 1 milliamp, with currents from about 50 femptoamps to about 100 microamps being preferred, and from about 1 picoamp to about 1 microamp being especially preferred.

In a preferred embodiment, the output signal is phase shifted in the AC component relative to the input signal. Without being bound by theory, it appears that the systems of the present invention may be

sufficiently uniform to allow phase-shifting based detection. That is, the complex biomolecules of the invention through which electron transfer occurs react to the AC input in a homogeneous manner, similar to standard electronic components, such that a phase shift can be determined. This may serve as the basis of detection between the presence and absence of the ETM, and/or differences between the presence of target-specific assay complexes comprising label probes and non-specific binding of the label probes to the system components.

The output signal is characteristic of the presence of the ETM; that is, the output signal is characteristic of the presence of the target-specific assay complex comprising label probes and ETMs. In a preferred embodiment, the basis of the detection is a difference in the faradaic impedance of the system as a result of the formation of the assay complex. Faradaic impedance is the impedance of the system between the electrode and the ETM. Faradaic impedance is quite different from the bulk or dielectric impedance, which is the impedance of the bulk solution between the electrodes. Many factors may change the faradaic impedance which may not effect the bulk impedance, and vice versa. Thus, the assay complexes comprising the nucleic acids in this system have a certain faradaic impedance, that will depend on the distance between the ETM and the electrode, their electronic properties, and the composition of the intervening medium, among other things. Of importance in the methods of the invention is that the faradaic impedance between the ETM and the electrode is signficantly different depending on whether the label probes containing the ETMs are specifically or non-specifically bound to the electrode.

Accordingly, the present invention further provides apparatus for the detection of nucleic acids using AC detection methods. The apparatus includes a test chamber which has at least a first measuring or sample electrode, and a second measuring or counter electrode. Three electrode systems are also useful. The first and second measuring electrodes are in contact with a test sample receiving region, such that in the presence of a liquid test sample, the two electrodes may be in electrical contact.

In a preferred embodiment, the first measuring electrode comprises a single stranded nucleic acid capture probe covalently attached via an attachment linker, and a monolayer comprising conductive oligomers, such as are described herein.

The apparatus further comprises an AC voltage source electrically connected to the test chamber; that is, to the measuring electrodes. Preferably, the AC voltage source is capable of delivering DC offset voltage as well.

In a preferred embodiment, the apparatus further comprises a processor capable of comparing the input signal and the output signal. The processor is coupled to the electrodes and configured to receive an output signal, and thus detect the presence of the target nucleic acid.

Thus, the compositions of the present invention may be used in a variety of research, clinical, quality control, or field testing settings.

In a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, or any of the others well known in the art.

In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly highly conserved HIV sequences. In addition, this allows direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, clymidia and other sexually transmitted diseases, may also be detected, for example using ribosomal RNA (rRNA) as the target sequences.

In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid (particularly rRNA), and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*, enterotoxic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the probes in an array are used for sequencing by hybridization.

Thus, the present invention provides for extremely specific and sensitive probes, which may, in some embodiments, detect target sequences without removal of unhybridized probe. This will be useful in the generation of automated gene probe assays.

Alternatively, the compositions of the invention are useful to detect successful gene amplification in PCR, thus allowing successful PCR reactions to be an indication of the presence or absence of a target sequence. PCR may be used in this manner in several ways. For example, in one embodiment, the PCR reaction is done as is known in the art, and then added to a composition of the invention comprising the target nucleic acid with a ETM, covalently attached to an electrode via a conductive oligomer with subsequent detection of the target sequence. Alternatively, PCR is done using nucleotides labelled with a ETM, either in the presence of, or with subsequent addition to, an electrode with a conductive oligomer and a target nucleic acid. Binding of the PCR product containing ETMs to the electrode composition will allow detection via electron transfer. Finally, the nucleic acid attached to the electrode via a conductive polymer may be one PCR primer, with addition of a second primer labelled with an ETM. Elongation results in double stranded nucleic acid with a ETM and electrode covalently attached. In this way, the present invention is used for PCR detection of target sequences.

In a preferred embodiment, the arrays are used for mRNA detection. A preferred embodiment utilizes either capture probes or capture extender probes that hybridize close to the 3' polyadenylation tail of the mRNAs. This allows the use of one species of target binding probe for detection, i.e. the probe contains a poly-T portion that will bind to the poly-A tail of the mRNA target. Generally, the probe will contain a second portion, preferably non-poly-T, that will bind to the detection probe (or other probe). This allows one target-binding probe to be made, and thus decreases the amount of different probe synthesis that is done.

In a preferred embodiment, the use of restriction enzymes and ligation methods allows the creation of "universal" arrays. In this embodiment, monolayers comprising capture probes that comprise restriction endonuclease ends, as is generally depicted in Figure 7. By utilizing complementary portions of nucleic acid, while leaving "sticky ends", an array comprising any number of restriction endonuclease sites is made. Treating a target sample with one or more of these restriction endonucleases allows the targets to bind to the array. This can be done without knowing the sequence of the target. The target sequences can be ligated, as desired, using standard methods such as ligases, and the target sequence detected, using either standard labels or the methods of the invention.

The present invention provides methods which can result in sensitive detection of nucleic acids. In a preferred embodiment, less than about 10 X 10^6 molecules are detected, with less than about 10 X 10^5 being preferred, less than 10 X 10^4 being particularly preferred, less than about 10 X 10^3 being especially preferred, and less than about 10 X 10^2 being most preferred. As will be appreciated by those in the art, this assumes a 1:1 correlation between target sequences and reporter molecules; if more than one reporter molecule (i.e. electron transfer moeity) is used for each target sequence, the sensitivity will go up.

While the limits of detection are currently being evaluated, based on the published electron transfer rate through DNA, which is roughly 1 X 10^6 electrons/sec/duplex for an 8 base pair separation (see Meade et al., Angw. Chem. Eng. Ed., 34:352 (1995)) and high driving forces, AC frequencies of about 100 kHz should be possible. As the preliminary results show, electron transfer through these systems is quite efficient, resulting in nearly 100 X 10^3 electrons/sec, resulting in potential femptoamp sensitivity for very few molecules.

In addition to the methods outlined herein, the invention further provides compositions, generally kits, useful in the practice of the invention. The kits include the compositions including the primers and enzymes, along with any number of reagents or buffers, including additional enzymes and primers, dNTPs and/or NTPs (including substituted nucleotides), buffers, salts, inhibitors, etc. The kits can optionally include instructions for the use of the kits.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

EXAMPLES

Example 1

Synthesis of nucleoside modified with ferrocene at the 2' position

The preparation of N6 is described.

Compound N1. Ferrocene (20 g, 108 mmol) and 4-bromobutyl chloride (20 g, 108 mmol) were dissolved in 450 mL dichloromethane followed by the addition of $AlCl_3$ anhydrous (14.7 g, 11 mmol). The reaction mixture was stirred at room temperature for 1 hour and 40 minutes, then was quenched by addition of 600 mL ice. The organic layer was separated and was washed with water until the

aqueous layer was close to neutral (pH = 5). The organic layer was dried with Na_2SO_4 and concentrated. The crude product was purified by flash chromatography eluting with 50/50 hexane/dichloromethane and later 30/70 hexane/dichloromethane on 300 g silica gel to afford 26.4 gm (73%) of the title product.

Compound N2. Compound N1 (6 g, 18 mmol) was dissolved in 120 mL toluene in a round bottom flask. zinc (35.9 g, 55 mmol), mercuric chloride (3.3g, 12 mmol) and water (100 mL) were added successively. Then HCl solution (12 M, 80 mL) was added dropwise. The reaction mixture was stirred at room temperature for 16 hours. The organic layer was separated, and washed with water (2 x 100 mL) and concentrated. Further purification by flash chromatography (hexane) on 270 gm of silica gel provided the desired product as a brown solid (3.3 g, 58%).

Compound N3. A mixture of 13.6 gm (51 mmol) of adenosine in 400 mL dry DMF was cooled in a ice-water bath for 10 minutes before the addition of 3.0 gm (76 mmol) of NaH (60%) . The reaction mixture was stirred at 0 °C for one hour before addition of **Compound N2** (16.4 g, 51 mmol). Then the temperature was slowly raised to 30 °C, and the reaction mixture was kept at this temperature for 4 hours before being quenched by 100 mL ice. The solvents were removed in vacuo. The resultant gum was dissolved in 300 mL water and 300 mL ethyl acetate. The aqueous layer was extracted thoroughly (3 x 300 mL ethyl acetate). The combined organic extracts were concentrated, and the crude product was purified by flash chromatography on 270 g silica gel. The column was eluted with 20%ethyl acetate/dichloromethane, 50 % ethyl acetate/dichloromethane, 70 % ethyl acetate/dichloromethane, ethyl acetate, 1 % methanol/ethyl acetate, 3 % methanol/ethyl acetate, and 5 % methanol/ethyl acetate. The concentration of the desired fractions provide the final product (6.5 g, 25%).

Compound N4. Compound N3 (6.5 g, 12.8 mmol) was dissolved in 150 mL dry pyridine, followed by adding TMSCl (5.6 g, 51.2 mmol) . The reaction mixture was stirred at room temperature for 1.5 hours. Then phenoxyacetyl chloride (3.3 g, 19.2 mmol) was added at 0 °C. The reaction was then stirred at room temperature for 4 hours and was quenched by the addition of 100 mL water at 0 °C. The solvents were removed under reduced pressure, and the crude gum was further purified by flash chromatography on 90 g of silica gel (1 % methanol/dichloromethane) (2.3 g, 28%).

Compound N5. Compound N4 (2.2 g, 3.4 mmol) and DMAP (200 mg, 1.6 mmol) were dissolved in 150 mL dry pyridine, followed by the addition of DMTCl (1.4 g, 4.1 mmol). The reaction was stirred under argon at room temperature overnight. The solvent was removed under reduced pressure, and the residue was dissolved in 250 mL dichloromethane. The organic solution was washed by 5% $NaHCO_3$ solution (3 x 250 mL) , dried over Na_2SO_4, and concentrated. Further purification by flash

chromatography on 55 g of silica gel (1 % TEA/50% hexane/dichloromethane) provided the desired product (1.3 g, 41%).

Compound N6. To a solution of **N5** (3.30 gm, 3.50 mmol) in 150 mL dichloromethane. Diisopropylethylamine (4.87 mL, 8.0 eq.) and catalytic amount of DMAP (200 mg) were added. The mixture was kept at 0 °C, and N, N-diisopropylamino cyanoethyl phosphonamidic chloride (2.34 mL, 10.48 mmol) was added. The reaction mixture was warmed up and stirred at room temperature overnight. After dilution by adding 150 mL of dichloromethane and 250 mL of 5 % $NaHCO_3$ aqueous solution, the organic layer was separated, washed with 5% NaHCO3 (250 mL), dried over Na_2SO_4, and concentrated. The crude product was purified on a flash column of 66 g of silica gel packed with 1% TEA in hexane. The eluting solvents were 1% TEA in hexane (500 mL), 1% TEA and 10% dichloromethane in hexane (500 mL), 1% TEA and 20% dichloromethane in hexane (500 mL). 1% TEA and 50% dichloromethane in hexane (500 mL). Fractions containing the desired products were collected and concentrated to afford the final product (3 gm, 75%).

Example 2

Synthesis of "Branched" nucleoside

The synthesis of **N17** is described, as depicted in Figure 11A.

Synthesis of N14. To a solution of *Tert*-butyldimethylsily chloride (33.38 g, 0.22 mol) in 300 mL of dichloromethane was added imidazole (37.69 g, 0.55 mol) . Immediately, large amount of precipitate was formed. 2-Bromoethanol (27.68 g, 0.22 mol,.) was added slowly at room temperature. The reaction mixture was stirred at this temperature for 3 hours. The organic layer was washed with water (200 mL), 5% $NaHCO_3$ (2 x 250 mL), and water (200 mL). The removal of solvent afforded 52.52 g of the title product (99%).

Synthesis of N15. To a suspension of adenosine (40 g, 0.15 mol) in 1.0 L of DMF at 0 °C, was added NaH (8.98 gm of 60% in mineral oil, 0.22 mol). The mixture was stirred at 0 °C for 1 hour, and **N14** (35.79 gm, 0.15mol) was added. The reaction was stirred at 30 °C overnight. It was quenched by 100 mL ice-water. The solvents were removed under high vaccum. The resultant foam was dissolved in a mixture of 800 mL of ethyl acetate and 700 mL of water. The aqueous layer was further extracted by ethyl acetate (3 x 200 mL). The combined organic layer was dried over Na_2SO_4 and concentrated. The crude product was further purified on a flash column of 300 g of silica gel packed with 1% TEA in dichloromethane. The eluting solvents were dichloromethane (500 mL), 3% MeOH in dichloromethane (500 mL), 5% MeOH in dichloromethane (500 mL), and 8% MeOH in

dichloromethane (2000 mL). The desired fractions were collected and concentrated to afford 11.70 g of the title product (19%).

Synthesis of N16. To a solution of **N15** (11.50 gm, 27.17 mmol) in 300 mL dry pyridine cooled at 0°C, was added trimethylsily chloride (13.71 mL, 0.11 mol, 4.0). The mixture was stirred at 0 °C for 40 min. Phenoxyacetyl chloride (9.38 mL, 67.93 mmol) was added. The reaction was stirred at 0 °C for 2.5 h. The mixture was then transferred to a mixture of 700 mL of dichloromethane and 500 mL water. The mixture was shaken well and organic layer was separated. After washing twice with 5% $NaHCO_3$ (2 x 300 mL), dichloromethane was removed on a rotovapor. Into the residue was added 200 mL of water, the resulting pyridine mixture was stirred at room temperature for 2 hours. The solvents were then removed under high vacuum. The gum product was co-evaporated with 100 mL of pyridine. The residue was dissolved in 250 mL of dry pyridine at 0 °C, and 4, 4'-dimethoxytrityl chloride (11.02 gm, 32.60 mmol) was added. The reaction was stirred at room temperature overnight. The solution was transferred to a mixture of 700 mL of dichloromethane and 500 mL of 5% $NaHCO_3$. After shaking well, the organic layer was separated, further washed with 5% $NaHCO_3$ (2 x 200 mL), and then concentrated. The crude product was purified on a flash column of 270 gm of silica gel packed with 1% TEA/30% CH_2Cl_2/Hexane. The eluting solvents were 1% TEA/ 50% CH_2Cl_2/Hexane (1000 mL), and 1% TEA /CH_2Cl_2 (2000 mL). The fractions containing the desired product were collected and concentrated to afford 10.0 g of the title product (43%).

Synthesis of N17. To asolution of **N16** (10.0 gm, 11.60 mmol) in 300 mL dichloromethane. Diisopropylethylamine (16.2 mL) and catalytic amount of N, N-dimethylaminopyridine(200 mg) were added. The mixture was cooled in an ice-water bath, and N, N-diisopropylamino cyanoethyl phosphonamidic chloride (7.78 mL, 34.82 mmol) was added. The reaction was stirred at room temperature overnight. The reaction mixture was diluted by adding 250 mL of dichloromethane and 250 mL of 5% $NaHCO_3$. After shaking well, the organic layer was separated and washed once more with the same amount of 5 % $NaHCO_3$ aqueous solution, dried over Na_2SO_4, and concentrated. The crude product was purified on a flash column of 120 gm of silica gel packed with 1% TEA and 10% dichloromethane in hexane. The eluting solvents were 1% TEA and 10% dichloromethane in hexane (500 mL), 1% TEA and 20% dichloromethane in hexane (500 mL), and 1% TEA and 40% dichloromethane in hexane (1500 mL). The right fractions were collected and concentrated to afford the final product (7.37gm, 60%).

The syntheses for two other nucleotides used for branching are shown in Figures 11B and 11C, with the Lev protecting group. These branching nucleotides branch from the phosphate, rather than the ribose (**N17**), and appear to give somewhat better results.

Example 3

Synthesis of triphosphate nucleotide containing an ETM

The synthesis of **AFTP** is described.

N3 (1.00 g,1.97 mmol) was dissolved in 15 mL of triethyl phosphate, followed by adding diisopropylethylamine (0.69 mL, 3.9 mmol). While the mixture was kept at 0 °C, and phospherous oxychloride (0.45g, 2.93 mmol) was added. The reaction mixture was stirred at 0 °C for 4 hours, then at 4 °C overnight. Bis(tributyl)ammonium phosphate (3.24 g, 5.91 mmol.) was added, and the reaction mixture was stirred at 0 °C for six hours, and at 4 °C overnight. The white precipitate produced in the reaction was removed by filtration. The filtrate was treated with water (20 mL), and yellow precipitate was formed. The precipitate was filtrated and was dried under high vacuum to afford 0.63 g of the title product as yellow solid.

Example 4

Synthesis of nucleoside with ferrocene attached via a phosphate

The synthesis of **Y63** is described.

Synthesis of C102: A reaction mixture consisting of 10.5gm (32.7 mmol) of **N2**, 16gm of potassium acetate and 350 ml of DMF was stirred at 100°C for 2.5hrs. The reaction mixture was allowed to cool to room temperature and then poured into a mixture of 400ml of ether and 800ml of water. The mixture was shaken and the organic layer was separated. The aqueous layer was extracted twice with ether. The combined ether extracts were dried over sodium sulfate and then concentrated for column chromatography. Silica gel(160 gm) was packed with 1% TEA/Hexane. The crude was loaded and the column was eluted with 1 % TEA/0-100 % CH_2Cl_2/Hexane. Fractions containing desired product were collected and concentrated to afford 5.8g (59.1 %) of **C102**.

Synthesis of Y61: To a flask containing 5.1gm (17.0 mmol) of **C102** was added 30ml of Dioxane. To this solution, small aliquots of 1M NaOH was added over a period of 2.5 hours or until hydrolysis was complete. After hydrolysis the product was extracted using hexane. The combined extracts were dried over sodium sulfate and concentrated for chromatography. Silica gel (100 gm) was packed in 10% EtOAc/ Hexane. The crude product solution was loaded and the column was eluted with 10% to 50% EtOAc in hexane. The fractions containing desired product were pooled and concentrated to afford 4.20 gm (96.1 %) of **Y61**.

Synth sis of Y62: To a flask containing 4.10 gm (15.9 mmol) of **Y61** was added 200ml of dichloromethane and 7.72 ml of DIPEA and 4.24 gm (15.9 mmol) of bis(diisopropylamino) chlorophosphine. This reaction mixture was stirred under the presence of argon overnight. After the reaction mixture was concentrated to 1/3 of its original volume, 200ml of hexane was added and then the reaction mixture was again concentrated to 1/3 is original volume. This procedure was repeated once more. The precipitated salts were filtered off and the solution was concentrated to afford 8.24gm of crude **Y62**. Without further purification, the product was used for next step.

Synthesis of Y63: A reaction mixture of 1.0 gm (1.45 mmol) of N-PAC deoxy-adenosine, 1.77g of the crude **Y62**, and 125mg of N, N-diisopropylammonium tetrazolide, and 100 ml of dichloromethane. The reaction mixture was stirred at room temperature overnight. The reaction mixture was then diluted by adding 100ml of CH_2Cl_2 and 100 mL of 5% $NaHCO_3$ solution. The organic phase was separated and dried over sodium sulfate. The solution was then concentrated for column chromatography. Silica gel (35 gm) was packed with 1 % TEA /Hexane. The crude material was eluted with 1 % TEA /10-40% CH_2Cl_2 / Hexane. The fractions containing product were pooled and concentrated to afford 0.25 gm of the title product.

Example 5

Synthesis of Ethylene Glycol Terminated Wire **W71**

Synthesis of W55: To a flask was added 7.5 gm (27.3 mmol) of *tert*-butyldiphenylchlorosilane, 25.0 gm (166.5 mmol) of tri(ethylene glycol) and 50 ml of dry DMF under argon. The mixture was stirred and cooled in an ice-water bath. To the flask was added dropwise a clear solution of 5.1 gm (30.0 mmol) of $AgNO_3$ in 80 mL of DMF through an additional funnel. After the completeness of addition, the mixture was allowed to warm up to room temperature and was stirred for additional 30 min. Brown AgCl precipitate was filtered out and washed with DMF(3 x 10 mL). The removal of solvent under reduced pressure resulted in formation of thick syrup-like liquid product that was dissolved in about 80 ml of CH_2Cl_2. The solution was washed with water (6 x 100 mL) in order to remove unreacted starting material, ie, tris (ethylene glycol), then dried over Na_2SO4. Removal of CH_2Cl_2 afforded ~ 10.5 g crude product, which was purified on a column containing 104 g of silica gel packed with 50 % CH_2Cl_2/hexane. The column was eluted with 3-5% MeOH/ CH_2Cl_2. The fractions containing the desired product were pooled and concentrated to afford 8.01 gm (75.5 %) of the pure title product.

Synthesis of W68: To a flask containing 8.01 gm (20.6.0 mmol) of **W55** was added 8.56 gm (25.8 mmol) of CBr_4 and 60 ml of CH_2Cl_2. The mixture was stirred in an ice-water bath. To the solution was slowly added 8.11 gm (31.0 mmol) of PPh_3 in 15 ml CH_2Cl_2. The mixture was stirred for about 35 min. at 0 °C , and allowed to warm to room temperature. The volume of the mixture was reduced to about

10.0 ml and 75 ml of ether was added. The precipitate was filtered out and washed with 2x75 of ether. Removal of ether gave about 15 gm of crude product that was used for purification. Silica gel (105 gm) was packed with hexane. Upon loading the sample solution, the column was eluted with 50 % CH_2Cl_2/hexane and then CH_2Cl_2. The desired fractions were pooled and concentrated to give 8.56gm (72.0 %) of pure title product.

Synthesis of W69: A solution of 5.2 gm (23.6 mmol)of 4-iodophenol in 50 ml of dry DMF was cooled in an ice-water bath under Ar. To the mixture was added 1.0 gm of NaH (60% in mineral oil, 25.0 mmol) portion by portion. The mixture was stirred at the same temperature for about 35 min. and at room temperature for 30 min. A solution of 8.68 gm (19.2 mmol) of **W68** in 20 ml of DMF was added to the flask under argon. The mixture was stirred at 50 °C for 12 hr with the flask covered with aluminum foil. DMF was removed under reduced pressure. The residue was dissolved in 300 ml of ethyl acetate, and the solution was washed with H_2O (6 x 50 mL). Ethyl acetate was removed under reduced pressure and the residue was loaded into a 100 g silica gel column packed with 30 % CH_2Cl_2/hexane for the purification. The column was eluted with 30-100% CH_2Cl_2/hexane. The fractions containing the desired product were pooled and concentrated to afford 9.5 gm (84.0 %) of the title product.

Synthesis of W70: To a 100 ml round bottom flask containing 6.89 gm (11.6 mmol) of **W69** was added 30 ml of 1M TBAF THF solution. The solution was stirred at room temperature for 5h. THF was removed and the residue was dissolved 150 ml of CH_2Cl_2. The solution was washed with H_2O (4 x 25 mL). Removal of solvent gave 10.5 gm of semi-solid. Silica gel (65 gm) was packed with 50 % CH_2Cl_2/hexane, upon loading the sample solution, the column was eluted with 0-3 % CH_3OH/CH_2Cl_2. The fractions were identified by TLC (CH_3OH : CH_2Cl_2 = 5 : 95). The fractions containing the desired product were collected and concentrated to afford 4.10 gm (99.0%) of the title product.

Synthesis of W71: To a flask was added 1.12 gm (3.18 mmol) of **W70**, 0.23 g (0.88 mmol) of PPh_3, 110 mg (0.19 mmol) of $Pd(dba)_2$, 110 mg (0.57 mmol) of CuI and 0.75g (3.2 mmol) of **Y4** (one unit wire). The flask was flushed with argon and then 65 ml of dry DMF was introduced, followed by 25 ml of diisopropylamine. The mixture was stirred at 55 °C for 2.5 h. All tsolvents were removed under reduced pressure. The residue was dissolved in 100 ml of CH_2Cl_2, and the solution was thoroughly washed with the saturated EDTA solution (2 x 100 mL). The Removal of CH_2Cl_2 gave 2.3 g of crude product. Silica gel (30 gm) was packed with 50 % CH_2Cl_2/hexane, upon loading the sample solution, the column was eluted with 10 % ethyl acetate/CH_2Cl_2. The concentration of the fractions containing the desired product gave1.35 gm (2.94 mmol) of the title product, which was further purified by recrystallization from hot hexane solution as colorless crystals.

Example 6

Synthesis of nucleoside attached to an insulator

Synthesis of C108: To a flask was added 2.0gm (3.67 mmol) of 2'-amino-5'-O-DMT uridine, 1.63gm (3.81 mmol) of **C44**, 5ml of TEA and 100ml of dichloromethane. This reaction mixture was stirred at room temperature over for 72hrs. The solvent was removed and dissolved in a small volume of CH_2Cl_2. Silica gel (35 gm) was packed with 2% CH_3OH/1% TEA/CH_2Cl_2, upon loading the sample solution, the column was eluted with the same solvent system. The fractions containing the desired product were pooled and concentrated to afford 2.5gm (80.4 %) of the title product.

Synthesis of C109: To a flask was added 2.4gm (2.80 mmol) of **C108**, 4ml of diisopropylethylamine and 80ml of CH_2Cl_2 under presence of argon. The reaction mixture was cooled in an ice-water bath. Once cooled, 2.10 gm (8.83 mmol) of 2-cyanoethyl diisopropylchloro-phosphoramidite was added. The mixture was then stirred overnight. The reaction mixture was diluted by adding 10ml of methanol and 150ml of CH_2Cl_2. This mixture was washed with a 5% $NaHCO_3$ solution, dried over sodium sulfate and then concentrated for column chromatography. A 65gm-silica gel column was packed in 1% TEA and Hexane. The crude product was loaded and the column was eluted with 1 % TEA/ 0-20 % CH_2Cl_2/Hexane. The fractions containing the desired product were pooled and concentrated to afford 2.69gm (90.9 %) of the title product.

Example 7

Comparison of Different ETM Attachments

A variety of different ETM attachments as depicted in Figure 1 were compared. As shown in Table 1, a detection probe was attached to the electrode surface (the sequence containing the wire in the table). Positive (i.e. probes complementary to the detection probe) and negative (i.e. probes not complementary to the detection probe) control label probes were added.

Electrodes containing the different compositions of the invention were made and used in AC detection methods. The experiments were run as follows. A DC offset voltage between the working (sample) electrode and the reference electrode was swept through the electrochemical potential of the ferrocene, typically from 0 to 500 mV. On top of the DC offset, an AC signal of variable amplitude and frequency was applied. The AC current at the excitation frequency was plotted versus the DC offset.

The results are shown in Table 2, with the Y63, VI and IV compounds showing the best results.

Metal Complexes	Redox Potential (mV)	10 Hz	100 Hz	1,000 Hz	10,000 Hz
I	400	Not clear	Not clear	Not clear	Not clear
II	350	0.15 μA	0.01 μA	0.005 μA	ND
III (+ control)	360	0.025 μA	0.085 μA	0.034 μA	ND
III (- control)	360	0.022 μA	0.080 μA	0.090 μA	ND
IV	140	0.34 μA	3.0 μA	13.0 μA	35 μA
V	400	0.02 μA	ND	0.15 μA	ND
VI(1)	140	0.22 μA	1.4 μA	4.4 μA	8.8 μA
VI(2)	140	0.22 μA	0.78 μA	5.1 μA	44 μA
VII	320	0.04 μA	ND	0.45 μA	No Peak
VIII(not purified)	360	0.047 μA	ND	ND	No Peak
Y63	160	.25 μA	ND	36 μA	130 μA

Not clear: There is no difference between positive control and negative control.
ND: Not determined

Table of the Oligonucleotides Containing Different Metal Complexes

Metal Complexes	Positive Control Sequence Containing Metal Complexes and Numbering	Negative Control Sequence Containing Metal Complexes and Numbering
I	5'-A(I)C (I)GA GTC CAT GGT-3' #D199_1	5'-A(I)G (I)CC TAG CTG GTG-3' #D200_1
II	5'-A(II)C (II)GA GTC CAT GGT-3' #D211_1,2	5'-A(II)G (II)CC TAG CTG GTG-3' #D212_1
III	5'-AAC AGA GTC CAT GGT-3' #D214_1	5'-ATG TCC TAG CTG GTG-3' #D57_1
IV	5'-A(IV)C (IV)GA GTC CAT GGT-3' #D215_1	5'-A(IV)G (IV)CC TAG CTG GTG-3' #D216_1
V	5'-A(V)C (V)GA GTC CAT GGT-3' #D203_1	5'-A(V)G (V)CC TAG CTG GTG-3' #D204_1
VI	5'-A(VI)C AGA GTC CAT GGT-3' #D205_1	5'-A(VI)G TCC TAG CTG GTG-3' #D206_1
VI	5'-A(VI)* AGA GTC CAT GGT-3' #D207_1	5'A(VI)* TCC TAG CTG GTG-3' #D208_1
VII	5'-A(VII)C (VII)GA GTC CAT GGT-3' #D158_3	5'-A(VII)G (VII)CC TAG CTG GTG-3' #D101_2

VIII	5'-A(VIII)C (VIII)GA GTC CAT GGT-3' #D217_1,2,3	5'-A(VIII)G (VIII)CC TAG CTG GTG-3' #D218_1

Metal Complexes	Sequence Containing Wire On G Surface and Numbering
I	5'-ACC ATG GAC TCT GT(U_w)-3' #D201_1,2
II	5-'ACC ATG GAC TCT GT(U_w)-3' #D201_1,2
III	5'-ACC ATG GAC TCT GT(U_w)-3' #D201_1,2
IV	5'-ACC ATG GAC TCT GT(U_w)-3' #D201_1,2
V	5'-ACC ATG GAC TCA GA(U_w)-3' #D83_17,18
VI	5'-ACC ATG GAC TCT GT(U_w)-3' #D201_1,2
VI	5'-ACC ATG GAC TCT GT(U_w)-3' #D201_1,2
VII	5'-ACC ATG GAC TCA GA(U_w)-3' #D83_17,18
VIII	5'-ACC ATG GAC TCA GA(U_w)-3' #D83_17,18

Example 8

Preferred Embodiments of the Invention

A variety of systems have been run and shown to work well, as outlined below. All compounds are referenced in Figure 19. Generally, the systems were run as follows. The surfaces were made, comprising the electrode, the capture probe attached via an attachment linker, the conductive oligomers, and the insulators, as outlined above. The other components of the system, including the target sequences, the capture extender probes, and the label probes, were mixed and generally annealed at 90°C for 5 minutes, and allowed to cool to room temperature for an hour. The mixtures were then added to the electrodes, and AC detection was done.

Use of a capture probe, a capture extender probe, an unlabeled target sequence and a label probe:

A capture probe **D112**, comprising a 25 base sequence, was mixed with the **Y5** conductive oligomer and the M44 insulator at a ratio of 2:2:1 using the methods of Example 16. A capture extender probe **D179**, comprising a 24 base sequence perfectly complementary to the **D112** capture probe, and a 24 base sequence perfectly complementary to the **2tar** target, separated by a single base, was added, with the **2tar** target. The

D179 molecule carries a ferrocene (using a C15 linkage to the base) at the end that is closest to the electrode. When the attachment linkers are conductive oligomers, the use of an ETM at or near this position allows verification that the **D179** molecule is present. A ferrocene at this position has a different redox potential than the ETMs used for detection. A label probe **D309** (dendrimer) was added, comprising a 18 base sequence perfectly complementary to a portion of the target sequence, a 13 base sequence linker and four ferrocenes attached using a branching configuration. A representative scan is shown in Figure 20A. When the **2tar** target was not added, a representative scan is shown in Figure 20B.

Use of a capture probe and a labeled target sequence:

Example A: A capture probe **D94** was added with the **Y5** and **M44** conductive oligomer at a 2:2:1 ratio with the total thiol concentration being 833 μM on the electrode surface, as outlined above. A target sequence (**D336**) comprising a 15 base sequence perfectly complementary to the **D94** capture probe, a 14 base linker sequence, and 6 ferrocenes linked via the **N6** compound was used. A representative scan is shown in Figure 20C. The use of a different capture probe, **D109**, that does not have homology with the target sequence, served as the negative control; a representative scan is shown in Figure 20D.

Example B: A capture probe **D94** was added with the **Y5** and **M44** conductive oligomer at a 2:2:1 ratio with the total thiol concentration being 833 μM on the electrode surface, as outlined above. A target sequence (**D429**) comprising a 15 base sequence perfectly complementary to the **D94** capture probe, a **C131** ethylene glycol linker hooked to 6 ferrocenes linked via the **N6** compound was used. A representative scan is shown in Figure 20E. The use of a different capture probe, **D109**, that does not have homology with the target sequence, served as the negative control; a representative scan is shown in Figure 20F.

Use of a capture probe, a capture extender probe, an unlabeled target sequence and two label probes with long linkers between the target binding sequence and the ETMs:

The capture probe **D112**, **Y5** conductive oligomer, the **M44** insulator, and capture extender probe **D179** were as outlined above. Two label probes were added: **D295** comprising an 18 base sequence perfectly complementary to a portion of the target sequence, a 15 base sequence linker and six ferrocenes attached using the **N6** linkage depicted in Figure 23. **D297** is the same, except that it's 18 base sequence hybridizes to a different portion of the target sequence. A representative scan is shown in Figure 20G. When the **2tar** target was not added, a representative scan is shown in Figure 20H.

Use of a capture probe, a capture extender probe, an unlabeled target sequence and two label probes with short linkers between the target binding sequence and the ETMs:

The capture probe **D112**, **Y5** conductive oligomer, the **M44** insulator, and capture extender probe **D179** were as outlined above. Two label probes were added: **D296** comprising an 18 base sequence perfectly complementary to a portion of the target sequence, a 5 base sequence linker and six ferrocenes attached

using the **N6** linkage depicted in Figure 23. **D298** is the same, except that it's 18 base sequence hybridizes to a different portion of the target sequence. A representative scan is shown in Figure 20I. When the **2tar** target was not added, a representative scan is shown in Figure 20J.

Use of two capture probes, two capture capture extender probes, an unlabeled large target sequence and two label probes with long linkers between the target binding sequence and the ETMs:

This test was directed to the detection of rRNA. The **Y5** conductive oligomer, the **M44** insulator, and one surface probe **D350** that was complementary to 2 capture sequences **D417** and **EU1** were used as outlined herein. The **D350**, **Y5** and **M44** was added at a **0.5:4.5:1** ratio. Two capture extender probes were used; **D417** that has 16 bases complementary to the **D350** capture probe and 21 bases complementary to the target sequence, and **EU1** that has 16 bases complementary to the **D350** capture probe and 23 bases complementary to a different portion of the target sequence. Two label probes were added: **D468** comprising a 30 base sequence perfectly complementary to a portion of the target sequence, a linker comprising three **glen** linkers as shown in Figure 19 (comprising polyethylene glycol) and six ferrocenes attached using **N6**. **D449** is the same, except that it's 28 base sequence hybridizes to a different portion of the target sequence, and the polyethylene glycol linker used (**C131**) is shorter. A representative scan is shown in Figure 20K.

Use of a capture probe, an unlabeled target, and a label probe:

Example A: A capture probe **D112**, **Y5** conductive oligomer and the **M44** insulator were put on the electrode at 2:2:1 ratio with the total thiol concentration being 833 µM. A target sequence **MT1** was added, that comprises a sequence complementary to **D112** and a 20 base sequence complementary to the label probe **D358** were combined; in this case, the label probe **D358** was added to the target sequence prior to the introduction to the electrode. The label probe contains six ferrocenes attached using the **N6** linkages depicted in Figure 23. A representative scan is shown in Figure 20L. The replacment of **MT1** with **NC112** which is not complementary to the capture probe resulted in no signal; similarly, the removal of **MT1** resulted in no signal.

Example B: A capture probe **D334**, **Y5** conductive oligomer and the **M44** insulator were put on the electrode at 2:2:1 ratio with the total thiol concentration being 833 µM. A target sequence **LP280** was added, that comprises a sequence complementary to the capture probe and a 20 base sequence complementary to the label probe **D335** were combined; in this case, the label probe **D335** was added to the target prior to introduction to the electrode. The label probe contains six ferrocenes attached using the **N6** linkages depicted in Figure 23. A representative scan is shown in Figure 20M. Replacing **LP280** with the **LN280** probe (which is complementary to the label probe but not the capture probe) resulted in no signal.

Example 9

Monitoring of PCR reactions using the invention

Monitoring of PCR r actions was done using an HIV sequence as the target sequence. Multiple reactions were run and stopped at 0 to 30 or 50 cycles. In this case, the sense primer contained the ETMs (using the N6 linkage described herein), although as will be appreciated by those in the art, triphosphate nucleotides containing ETMs could be used to label non-primer sequences. The surface probe was designed to hybridize to 16 nucleotides of non-primer sequences, immediately adjacent to the primer sequence; that is, the labeled primer sequence will not bind to the surface probe. Thus, only if amplification has occured, such that the amplified sequence will bind to the surface probe, will the detection of the adjacent ETMs proceed.

The target sequence in this case was the plasmid pBKBH10S (NIH AIDS Research and Reference Reagent program - McKesson Bioservices, Rockville MD) which contains an 8.9 kb SstI fragment of pBH10-R3 dervied from the HXB2 clone which contains the entire HIV-1 genome and has the Genbank accession code K03455 or M38432) inserted into the SstI site on pBluescript II-KS(+). The insert is oriented such that transcription from the T7 promoter produces sense RNA.

The "sense" primer, **D353**, was as follows: 5'-(N6)A(N6)AGGGCTGTTGGAAATGTGG-3'. The "antisense" primer, **D351**, was as follows: 5'-TGTTGGCTCTGGTCTGCTCTGA-3'. The following is the expected PCR product of the reaction, comprising 140 bp:

5'-(N6)A(N6)AGGGCTGTTGGAAATGTGGAAAGGAAGGACACCAAATGAAGATTGTACTGAGAGACAGGCT

3'-TTTTTCCCGACAACCTTTACACCTTTCCTTCCTGTGGTTTACTTTCTAACATGACTCTCTGTCCGA

AATTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATTTTCTTCAGAGCAGACCAGAGC

TTAAAAAATCCCTTCTAGACCGGAAGGATGTTCCCTTCCGGTCCCTTAAAAGAAGTCTCGTCTGGTCTCG

CAACA-3'

GTTTG-5'

The surface capture probe (without any overlap to the sense primer) **D459** was as follows: 5'-TTGGTGTCCTTCCTTU-4 unit wire(C11)-3'.

PCR reaction conditions were standard: TAQ polymerase at TAQ 10X buffer. 1 μM of the primers was added to either 6 X 10^3, 6X 10^6 or 6 X 10^7 molecules of template. The reaction conditions were 90°C for 30 sec, 57°C for 30 sec, and 70°C for 1 minute.

The electrodes were prepared by melting 0.127 mm diamter pure gold wire on one end to form a ball. The electrodes were dipped in aqua regia for 20 seconds and tehn rinse with water. The SAM was deposited by dipping the electrode into a deposition solution of 1.3:4.0:7 D459:H6:M44 in 37:39:24 THF:ACN:water at 1 mM total thiol which was heated at 50°C for five minutes prior to the introduction of the electrodes. The electrodes

were added and then removed immediately to room temperature to sit for 15 minutes. Electrodes were then transferred to M44 (in 37:39:24 THF:ACN:water at 400 μM total thiol conc ntration). The electrodes sat in M44 at room tem for 5 minutes, then the following heat cycling was applied: 70°C for 1 minute, followed by 55°C for 30 sec, repeating this cycle 2 more times followed by a 0.3 °C ramp down to RT with soaking at RT for 10 minutes. The electrodes were taken out of M44 solution, rinsed in 2XSSC, and hybridized as follows. The PCR products were adjusted to 6XSSC (no FCS). The control was also adjusted to 6XSSC. Hybridization was carried out at RT after rinsing twice in 6XSSC for at least 1.5 hours. ACV conditions were as follows: Ag/AgCl reference electrode and Pt auxillary electrodes were used, and $NaClO_4$ was used as the electrolyte solution. ACV measurements were carried out as follows: v=10 Hz, ϵ=25 mV, scan range -100 mV to 500 mV. The data is shown in Figure 26.

Example 10

Ligation on an Electrode Surface

The design of the experiment is shown in Figure 21, for the detection of an HIV sequence. Basically, a surface probe **D368** (5'-(H2)CCTTCCTTTCCACAU-4 unit wire(C11)-3') was attached to an electrode comprising M44 and **H6** (H6 is a two unit wire terminating in an acetylene bond) at a ratio of **D368**:**H6**:M44 of 1:4:1 with a total thiol concentration of 833 μM. A ligation probe **HIVLIG** (5'-CCACCAGATCTTCCCTAA AAAATTAGCCTGTCTCTCAGTACAATCTTTCATTTGGTGT-3') and the target sequence **HIVCOMP** (5'-ATGTGGAAAGAAAGGACACCAATTGAAAGATTGTACTGAGAGACAGGCTAATTTTTTTAGGGAAGATCTGG-3') was added, with ligase and the reaction allowed to proceed. The reaction conditions were as follows: 10 μM of **HIVLIG** annealed to **HIVCOMP** were hybridized to the electrode surface (in 6XSSC) for 80 min. The surface was rinsed in ligase buffer. The ligase (T4) and buffer were added and incubated for 2 hours at RT. Triton X at 10^{-4} M was added at 70°C to allow the denaturation of the newly formed hybridization complex, resulting in the newly formed long surface probe (comprising **D368** ligated to the **HIVLIG** probe). The addition of the **D456** signalling probe (5'-(N6)G(N6)CT(N60C(N60G(N6)C(N6)TTCTGCACCGTAAGCCA TCAAAGATTGTACTGAG-3') allowed detection (results not shown). The **D456** probe was designed such that it hybridizes to the HIVLIG probe; that is, a surface probe that was not ligated would not allow detection.

Example 11

Use of capture probes comprising ethylene glycol linkers

The capture probe for a rRNA assay containing 0, 4 and 8 ethylene glycol units was tested on four separate electrode surfaces. Surface 1 contained 2:1 ratio of **H6**:M44, with a total thiol concentration of 500 μM. Surface 2 contained a 2:2:1 ratio of **D568/H6/**M44 with a total thiol concentration of 833 μM. Surface 3 contained a 2:2:1 ratio of **D570/H6/**M44 with a total thiol concentration of 833 μM. **D568** was a capture probe comprising 5'-GTC AAT GAG CAA AGG TAT TAA (P282)-3'. **P282** was a thiol. **D569** was a capture probe

comprising 4 ethylene glycol units: 5'-GTC AAT GAG CAA AGG TAT TAA **(C131) (P282)**-3'. **D570** was a capture probe comprising 8 ethylene glycol units: 5'-GTC AAT GAG CAA AGG TAT TAA **(C131) (C131) (P282)**-3'. The **H6** (in the protected form) was as follows: $(CH_3)_3Si$-$(CH_2)_2$-S-(C_6H_5)-C≡C-(C_6H_5)-C≡CH. **M44** is the same as **M43** and was as follows: HS-$(CH_2)_{11}$-$(OCH_2CH_3)_3$-OH. The **D483** label probe hybridizes to a second portion of the rRNA target, and was as follows: 5'-(N6)C(N6) G(N6C (N6)GG CCT (N6)C(N6) G(N6)C (N6)(C131)(C131) (C131)(C131)T TAA TAC CTT TGC TC-3'. The **D495** is a negative control and was as follows: 5'-GAC CAG CTA GGG ATC GTC GCC TAG GTGAG(C131) (C131)(C131)(C131) (N6)G(N6) CT(N6) C(N6)G (N6)C(N6)-3'. The results were as follows:

Surface 1:	**D483**	~0 (no capture probe present)
	D495	0
Surface 2:	**D483**	126 nA
	D495	1.29 nA
Surface 3:	**D483**	19.39 nA
	D495	1.51 nA
Surface 4:	**D483**	84 nA
	D495	1.97 nA

As is shown, the system is working well.

Example 12

Detection of rRNA and a Comparison of Different Amounts of ETMs

The most sensitive rRNA detection to date used **D350/H6/M44** surfaces mixed in a ration of 1:3.5:1.5 deposited at a 833 μM total thiol concentration. **D350** is a 4 unit wire with a 15mer DNA; **H6** is a 2 unit wire; and **M44** is an ethylene glycol terminated alkane chain. Better detection limites are seen when the target molecule is tethered to the sensor surface at more than one place. To date, two tether points have been used. A **D417** tether sequence (42mer) and a **EU1** capture sequence (62mer) bound the 16S rRNA to the **D350** on the surface. A series of 9 label probes (**D449, D469, D489, D490, D491, D476, D475 and D477)** pre-annealed to the rRNA gave the electrochemical signal. These label probes (signalling molecules) have 6 or 8 **N6** or **Y63** type ferrocenes. The label probes that flank the tack-down regions were replaced (one end at a time) with label probes containing either 20 or 40 ferrocenes. Additionally, a label probe that binds to a region in the middle of the tack-down regions was replaced with label probes containing either 20 or 40 ferrocenes. When 2 6-ferrocene containing label probes were replaced by 2 40-ferrocene containing label probes, there was a 12-fold increase in the positive signal. The non-specific signal went up as well, exhibiting a 1.5 increase in the signal to noise ratio. Currently the best system utilizes tacking down the rRNA in two places and used a 40-ferrocene label probe to flank the 3' tack down point and bind the remaining face of the rRNA molecule with

6-ferrocene containing label probes. Additional tack down points, and a plurality of label probes, is contemplated.

A typical experimental protocol is as follows:

Surface derivatization: 20 µL of deposition solution (1:3.5:1.5 of D350:H6:M44 at total thiol concentration of 833 µM in 43.2% THF, 45.9% ACN, 10.9 % H20) was heated in a closed half milliliter eppendorf tube at 50°C for 5 minutes. A melted gold ball electrode was inserted into the solution and then moved immediately to room temperature to incubate for 15 minutes. The electrode was then transferred into ~200 µL of 400 µM M44 in 37% TH, 39% ACN, 24% H2O, where it incubated for 5 minutes at room temperature, 2 minutes at 40°C, 2 minutes at 30°C, and then an additional 15 minutes at room temperature. The electrode was then briefly dipped in 2X SSC (aqueous buffered salt solution) and hybridized as below.

Hybridization solutions were annealed by heating at 70°C for 30 seconds and then cooling to 22°C over ~ 38 seconds. The molecules were all in 4X SSC at twice the targeted concentrations, with the rRNA at 35 U.S.C. § µM, the capture sequence at 1.0 µM, and the label probes at 3 µM. After annealing, the solution was diluted 1:1 with fetal calf serum, halving the concentrations and changing the solvent to 2X SSC with 50% FCS. It should be noted that a recent experiment with model compounds suggest that a dilution by 1.2 with bovine serum albumin may be desirable: the reduction in non-specific binding was the same, but the sample concentration is not diluted and the positive signal was enhanced by a factor of 1.5. This was not done using the rRNA target, however. Solutions were aliquotted into 20 µL volumes for hybridization.

Hybridization was done as follows: After the 2X SSC dip described above, the derivatized electrode was placed into an eppendorf tube with 20 µL hybridization solution. It was allowed to hybridize at room temperature for 10 minutes.

Immediately before measurement, the electrode was briefly dipped in room temperature 2X SSC. It was then transferred into the 1 M $NaClO_4$ electrolyte and an alternating current voltammogram was taken with an applied alternating current of 10 Hz frequency and a 25 mV center-to-peak amplitude.

10 basic experiments were run (system components in parentheses):

System 1. rRNA is tacked down at only one point (D449 + D417(EU2) + D468

System 2. rRNA is tacked down at two points

System 3. two point tack down plus two label probes comprising 20 ferrocenes each directed to a flanking region of the second tack down point

System 4. two point tack down plus two label probes comprising 40 ferrocenes each directed to a flanking region of the second tack down point

System 5. two point tack down plus two label probes comprising 20 ferrocenes each directed to a flanking region of the first tack down point

System 6. two point tack down plus two label probes comprising 40 ferrocenes each directed to a flanking region of the first tack down point

System 7. two point tack down plus a label probe comprising 25 bases that binds to the middle region (i.e. the region between the two tack down points) containing 20 ferrocenes.

System 8. two point tack down plus a label probe comprising 25 bases that binds to the middle region (i.e. the region between the two tack down points) containing 40 ferrocenes.

System 9. two point tack down plus a label probe comprising 40 bases that binds to the middle region (i.e. the region between the two tack down points) containing 20 ferrocenes.

System 10. two point tack down plus a label probe comprising 40 bases that binds to the middle region (i.e. the region between the two tack down points) containing 40 ferrocenes.

The results are shown in Figure 22. It is clear from the results that multipoint tethering of large targets is better than single point tethering. More ETMs give larger signals, but require more binding energy; 35 bases of recognition to the target.

Example 13

Direct Comparison of Different Configurations of Ferrocenes

A comparison of different configurations of ferrocene was done, as is generally depicted in Figure 23. Figures 23A, 23B, 23C and 23D schematically depict the orientation of several label probes. **D94** was as follows: 5'-ACC ATG CAC ACA GA(C11)-3'. **D109** was as follows: 5'-CTG CGG TTA TTA AC(C11)-3'. The "+" surface was a 2:2:1 ratio of **D94:H6:M44**, with a total thiol concentration of 833 µM. The "-" surface was a 2:2:1 ratio of **D109:H6:M44**, with a total thiol concentration of 833 µM. The **D548** structure was as follows: 5'-(N38)(N38)(N38) (N38)(N38)(N38) (N38)(N38)(N38) ATC TGT GTC CAT GGT-3'. On each N38 was a 5'-

(H2)(C23)-3'. The **D549** structure was as follows: 5'-(N38)(N38)(N38) (N38)(N38)(N38) (N38)(N38)(N38) ATC TGT GTC CAT GGT-3'. On each N38 was a 5'-(H2)(C23)(C23)-3'.

The **D550** structure was as follows: 5'-(N38)(N38)(N38) (N38) AT CTG TGT CCA TGG T-3'. On each N38 was a 5'-(H2)(C23)(C23)-3'. The **D551** structure was as follows: 5'-(n38)(N38)(N38) (N38)ATCTG TGT CAA TGG T-3'. On each N38 was a 5'-(H2)(C23)(C23)(C23)(C23)-3'. A 5' N38 has two sites for secondary modification. A representative schematic is shown in Figure 23E.

The results, shown in the figures, show that the **D551** label probes gave the highest signals, with excellent signal-to-noise ratios.

Example 17

Ferrocene polymers as both recruitment linker and ETM

This system is shown in Figure 25. **D405** has the structure: 5'-(C23)(C23)(C23) (C23)(C23)(C23) (C23)(C23)(C23) (C23)AT CTG TGT CCA TGG T-3'. The system was run with two surfaces: the "+" surface was a 2:2:1 ratio of **D94:H6:M44**, with a total thiol concentration of 833 µM. The "-" surface was a 2:2:1 ratio of **D109:H6:M44**, with a total thiol concentration of 833 µM. The results, shown in Figure 25B, show that the system gave a good signal in the presence of a complementary capture probe.

CLAIMS

We claim:

1. A method for detecting a first target nucleic acid sequence comprising:
a) hybridizing at least a first primer nucleic acid to said first target sequence to form a first hybridization complex;
b) contacting said first hybridization complex with a first enzyme to form a modified first primer nucleic acid;
c) disassociating said first hybridization complex;
d) forming a first assay complex comprising at least one ETM and said modified first primer nucleic acid, wherein said first assay complex is covalently attached to an electrode; and
e) detecting electron transfer between said ETM and said electrode as an indication of the presence of said target sequence.

2. A method according to claim 1 wherein steps a) through c) are repeated prior to step d).

3. A method according to claim 1 or 2 further comprising:
a) hybridizing at least a second primer nucleic acid to a second target sequence that is substantially complementary to said first target sequence to form a second hybridization complex;
b) contacting said second hybridization complex with said first enzyme to form a modified second primer nucleic acid;
c) disassociating said second hybridization complex; and
d) forming a second assay complex comprising at least one ETM and said modified second primer nucleic acid, wherein said second assay complex is covalently attached to an electrode.

4. A method according to claim 1 or 3 wherein steps a) through c) are repeated prior to step d).

5. A method according to claim 1, 2, 3 or 4 wherein said first enzyme comprises a DNA polymerase and said modification is an extension of said primer such that the polymerase chain reaction (PCR) occurs.

6. A method according to claim 1, 2, 3 or 4 wherein said first enzyme comprises a ligase and said modification comprises a ligation of said first primer which hybridizes to a first domain of said first target sequence to a third primer which hybridizes to a second adjacent domain of said first target sequence, such that the ligase chain reaction (LCR) occurs.

7. A method according to claim 3, 4 or 7 wherein said first enzyme comprises a ligase and said modification is a ligation of said second primer which hybridizes to a first domain of said second target sequence to a fourth

primer which hybridizes to a second adjacent domain of said second target sequence, such that the ligase chain reaction (LCR) occurs.

8. A method according to claim 1 or 2 wherein said first primer comprises a first probe sequence, a first scissile linkage and a second probe sequence, wherein said first enzyme will cleave said first scissile linkage resulting in the separation of said first and said second probe sequences and the disassociation of said first hybridization complex, leaving said first target sequence intact, such that the cycling probe technology (CPT) reaction occurs.

9. A method according to claim 3, 4 or 9 wherein said second primer comprises a third probe sequence, a second scissile linkage and a fourth probe sequence, wherein said first enzyme will cleave said second scissile linkage resulting in the separation of said third and said fourth probe sequences and the disassociation of said second hybridization complex, leaving said second target sequence intact, such that the cycling probe technology (CPT) reaction occurs.

10. A method according to claim 1 or 2 wherein said first enzyme is a polymerase that extends said first primer and said modified first primer comprises a first newly synthesized strand, and said method further comprises:

a) the addition of a second enzyme comprising a nicking enzyme that nicks said extended first primer leaving said first target sequence intact; and

b) extending from said nick using said polymerase, thereby displacing said first newly synthesized strand and generating a second newly synthesized strand;

such that strand displacement amplification (SDA) occurs.

11. A method according to claim 3, 4 or 10 wherein said first enzyme is a polymerase that extends said second primer and said modified first primer comprises a third newly synthesized strand, and said method further comprises:

a) the addition of a second enzyme comprising a nicking enzyme that nicks said extended second primer leaving said second target sequence intact; and

b) extending from said nick using said polymerase, thereby displacing said third newly synthesized strand and generating a fourth newly synthesized strand;

such that strand displacement amplification (SDA) occurs.

12. A method according to claim 1 or 2 wherein said first target sequence is a RNA target sequence, said first primer nucleic acid is a DNA primer comprising an RNA polymerase promoter, said first enzyme is a reverse-transcriptase that extends said first primer to form a first newly synthesized DNA strand, and said method further comprises:

a) the addition of a second enzyme comprising an RNA degrading enzyme that degrades said first target sequence;

b) the addition of a third primer that hybridizes to said first newly synthesized DNA strand;

c) the addition of a third enzyme comprising a DNA polymerase that extends said third primer to form a second newly synthesized DNA strand, to form a newly synthesized DNA hybrid;

d) the addition of a fourth enzyme comprising an RNA polymerase that recognizes said RNA polymerase promoter and generates at least one newly synthesized RNA strand from said DNA hybrid;

such that nucleic acid sequence-based amplification (NASBA) occurs.

13. A method for detecting a target nucleic acid sequence comprising:

a) forming a first hybridization complex comprising an amplifier probe and a target sequence, wherein said amplifier probe comprises at least two amplification sequences;

b) hybridizing a first portion of at least one label probe to all or part of at least one amplification sequence;

c) hybridizing a second portion of said label probe to a detection probe covalently attached to an electrode via a conductive oligomer to form a second hybridization complex that contains at least a first electron transfer moiety (ETM); and

d) detecting said label probe by measuring electron transfer between said first ETM and said electrode.

14. A method for detecting a target nucleic acid sequence comprising:

a) forming a first hybridization complex comprising an amplifier probe and a target sequence, wherein said amplifier probe comprises at least two amplification sequences, wherein said first hybridization complex is covalently attached to an electrode comprising a monolayer comprising conductive oligomers;

b) hybridizing at least one label probe comprising at least one electron transfer moiety (ETM) to all or part of at least one amplification sequence;

c) detecting said label probe by measuring electron transfer between said first ETM and said electrode.

15. A kit for the detection of a first target nucleic acid sequence comprising:

a) at least a first nucleic acid primer substantially complementary to at least a first domain of said target sequence;

b) at least a first enzyme that will modify said first nucleic acid primer; and

c) an electrode comprising at least one detection probe covalently attached to said electrode via a conductive oligomer.

16. A kit for the detection of a first target nucleic acid sequence comprising:

a) at least a first nucleic acid primer substantially complementary to at least a first domain of said target sequence;

b) at least a first enzyme that will modify said first nucleic acid primer; and

c) an electrode comprising a monolayer comprising conductive oligomers.

17. A kit according to claim 15 or 16 for the detection of a PCR reaction wherein said first enzyme is a thermostabile DNA polymerase.

18. A kit according to claim 15 or 16 for the detection of a LCR reaction wherein said first enzyme is a ligase and said kit comprises a first nucleic acid primer substantially complementary to a first domain of said first target sequence and a third nucleic acid primer substantially complementary to a second adjacent domain of said first target sequence.

19. A kit according to claim 15 or 16 for the detection of a strand displacement amplification (SDA) reaction wherein said first enzyme is a polymerase and said kit further comprises a nicking enzyme.

20. A kit according to claim 15 or 16 for the detection of a NASBA reaction wherein said first enzyme is a reverse transcriptase, and said kit comprises a second enzyme comprising an RNA degrading enzyme, a third primer, a third enzyme comprising a DNA polymerase and a fourth enzyme comprising an RNA polymerase.

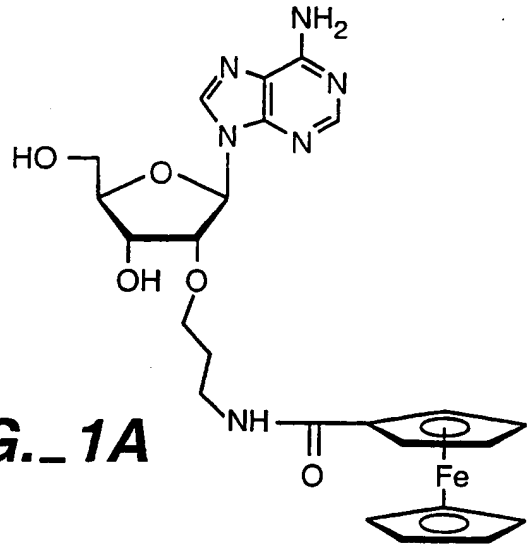


FIG._1A

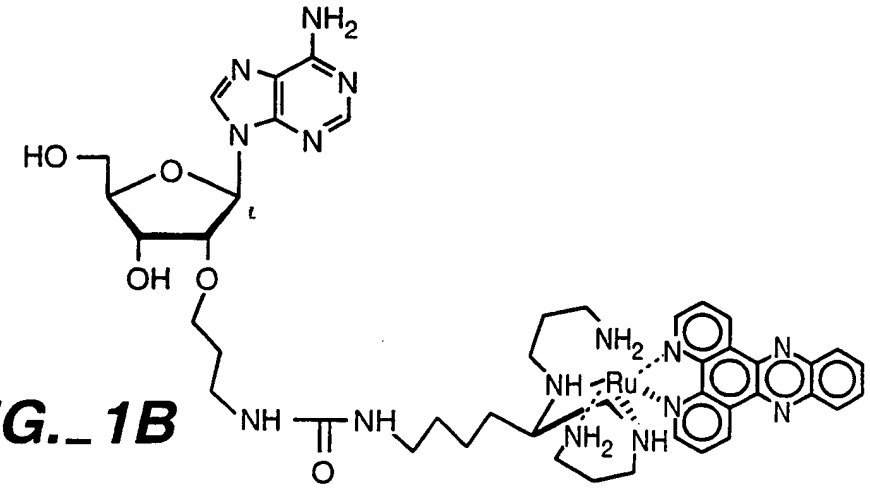


FIG._1B

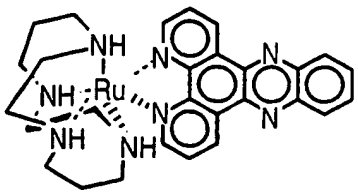


FIG._1C

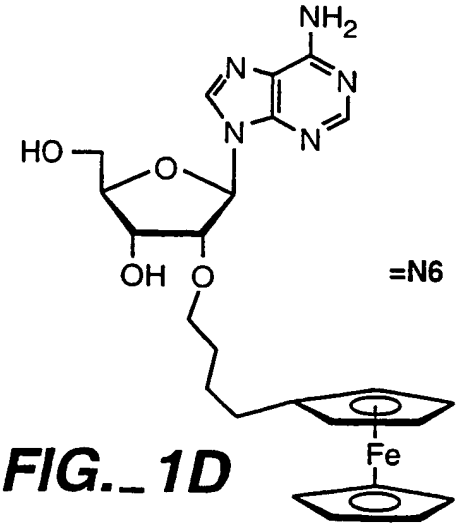


FIG._1D

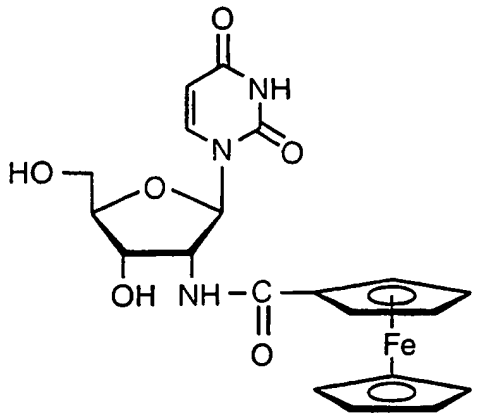
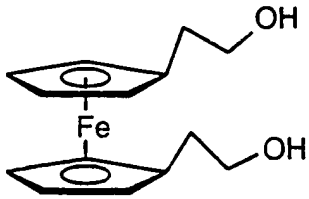


FIG._1E



=C23

FIG._1F

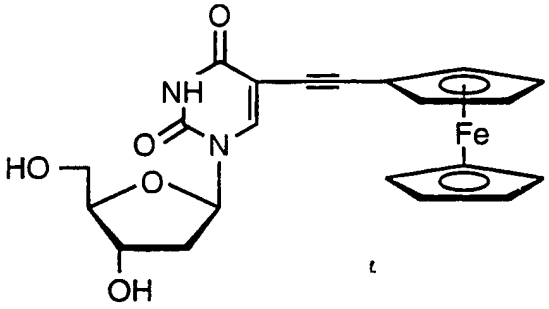


FIG._1G

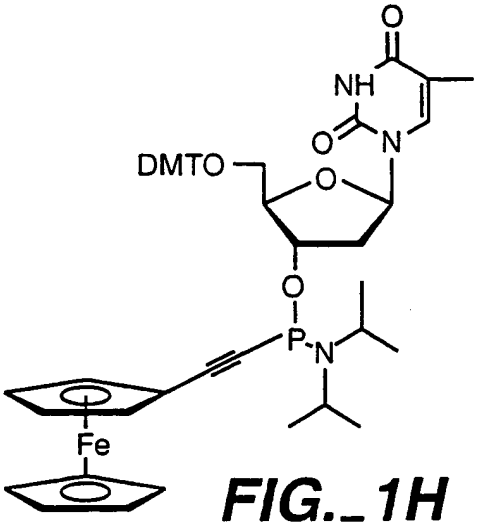
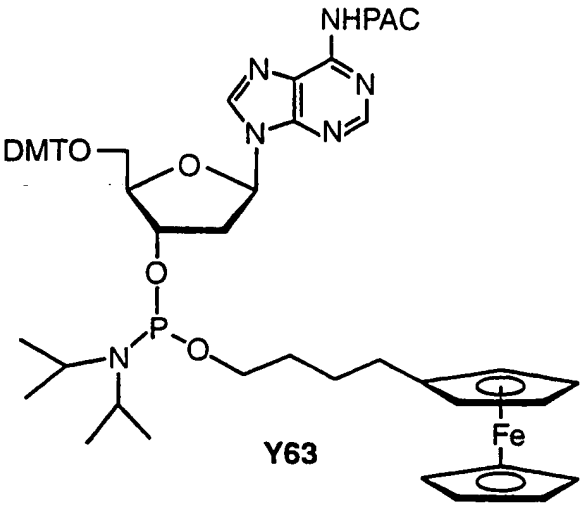


FIG._1H



Y63

FIG._1I

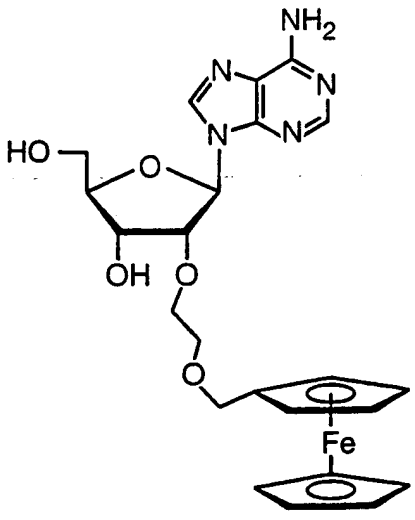


FIG._1J

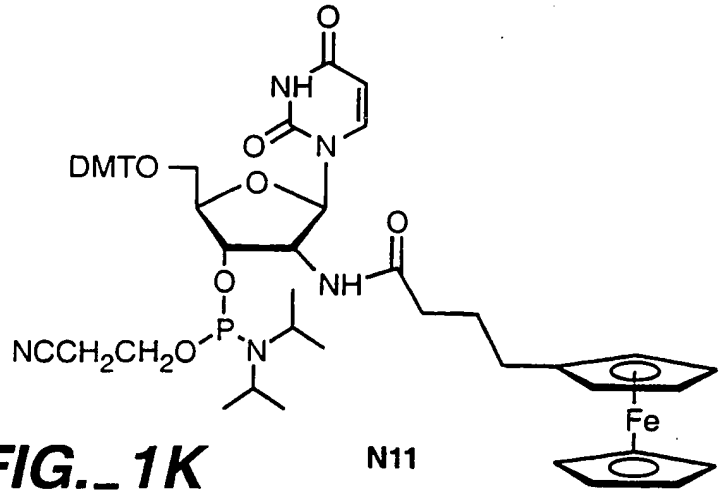
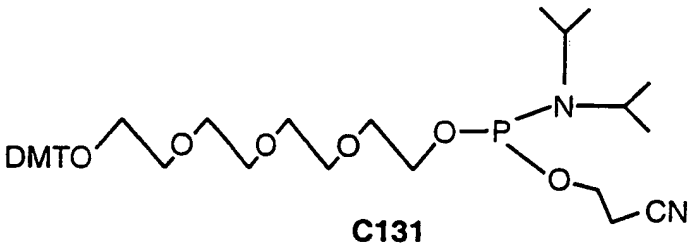


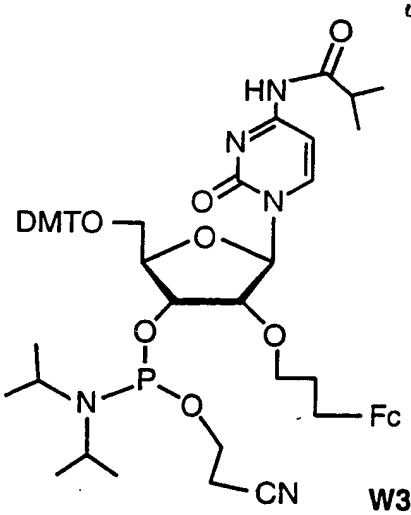
FIG._1K

N11



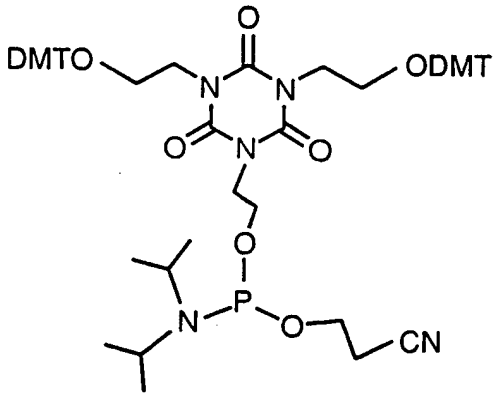
C131

FIG._1L



W38

FIG._1M



BRANCHING

FIG._1N

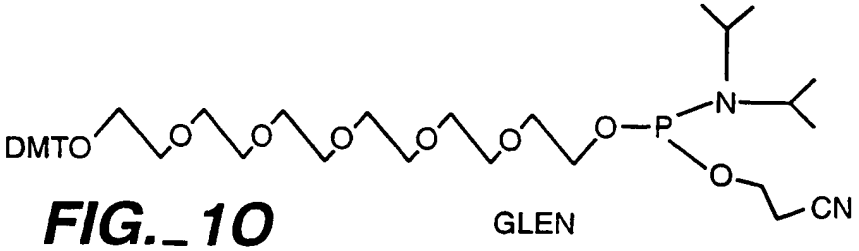


FIG._1O

GLEN

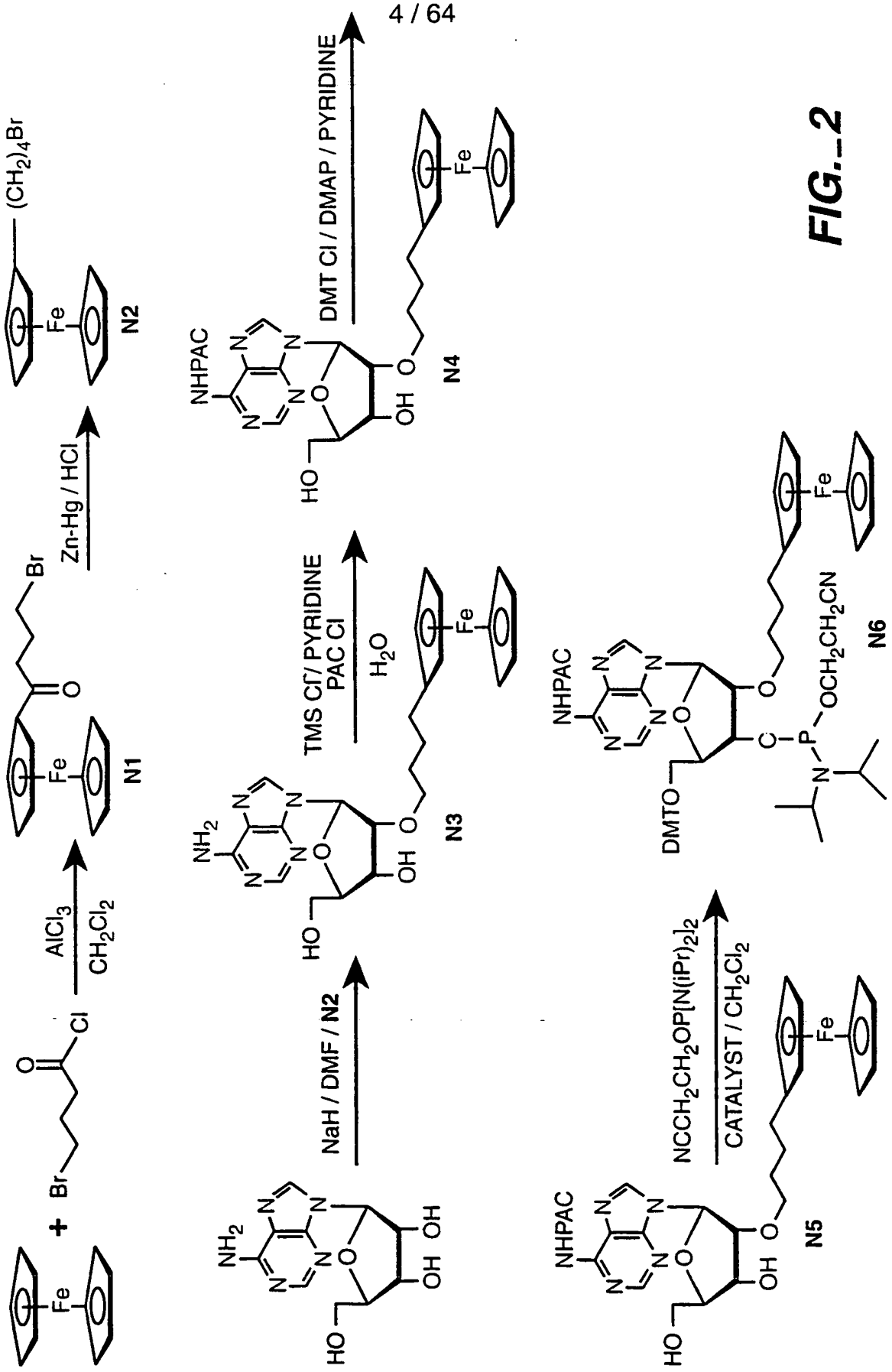


FIG._2

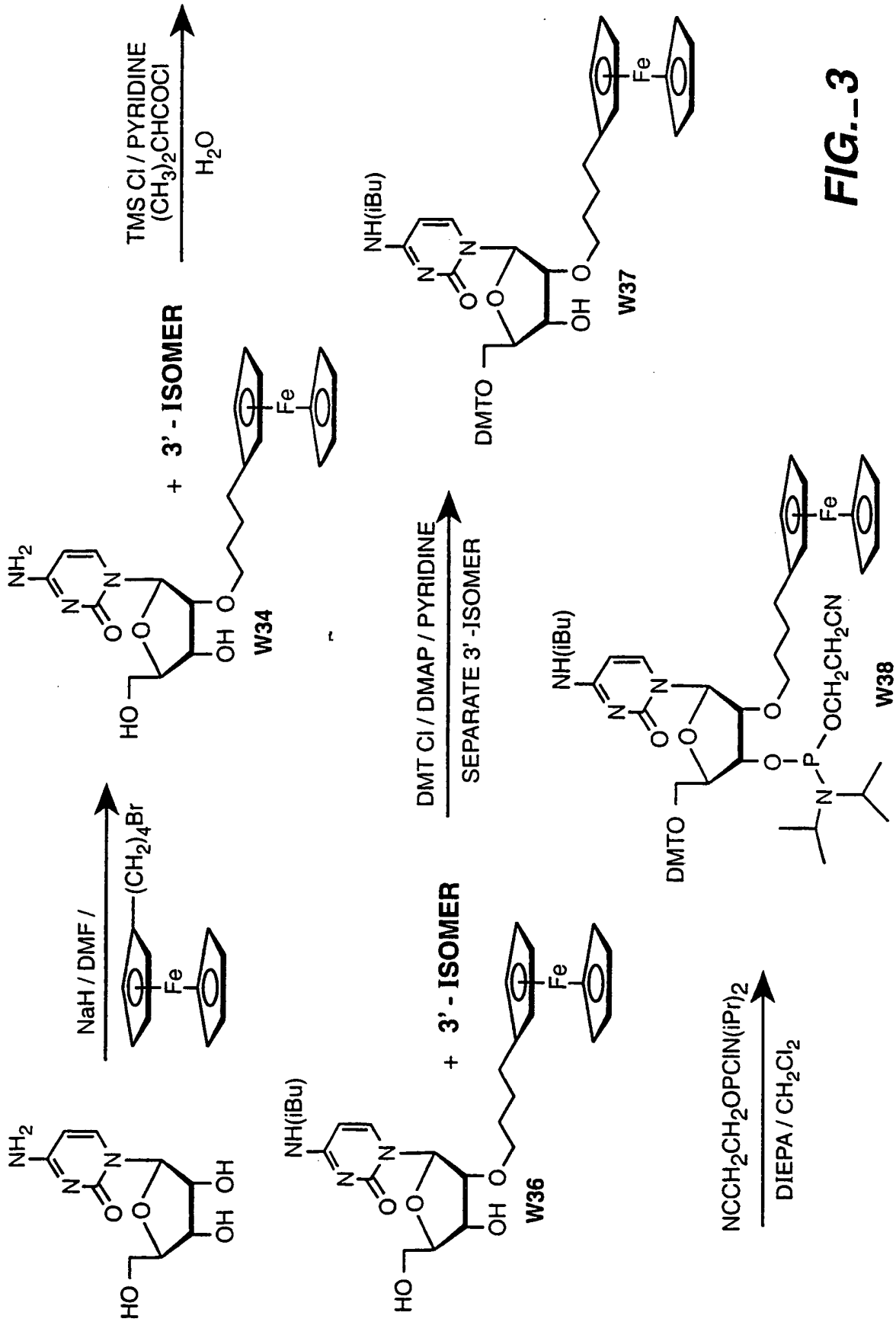

TMS Cl / PYRIDINE
(CH3)2CHCOCl
H2O
+ 3' - ISOMER
W34
W37
DMT Cl / DMAP / PYRIDINE
SEPARATE 3' -ISOMER
NaH / DMF /
(CH2)4Br
+ 3' - ISOMER
W36
W38
NCCH2CH2OPClN(iPr)2
DIEPA / CH2Cl2

FIG._3

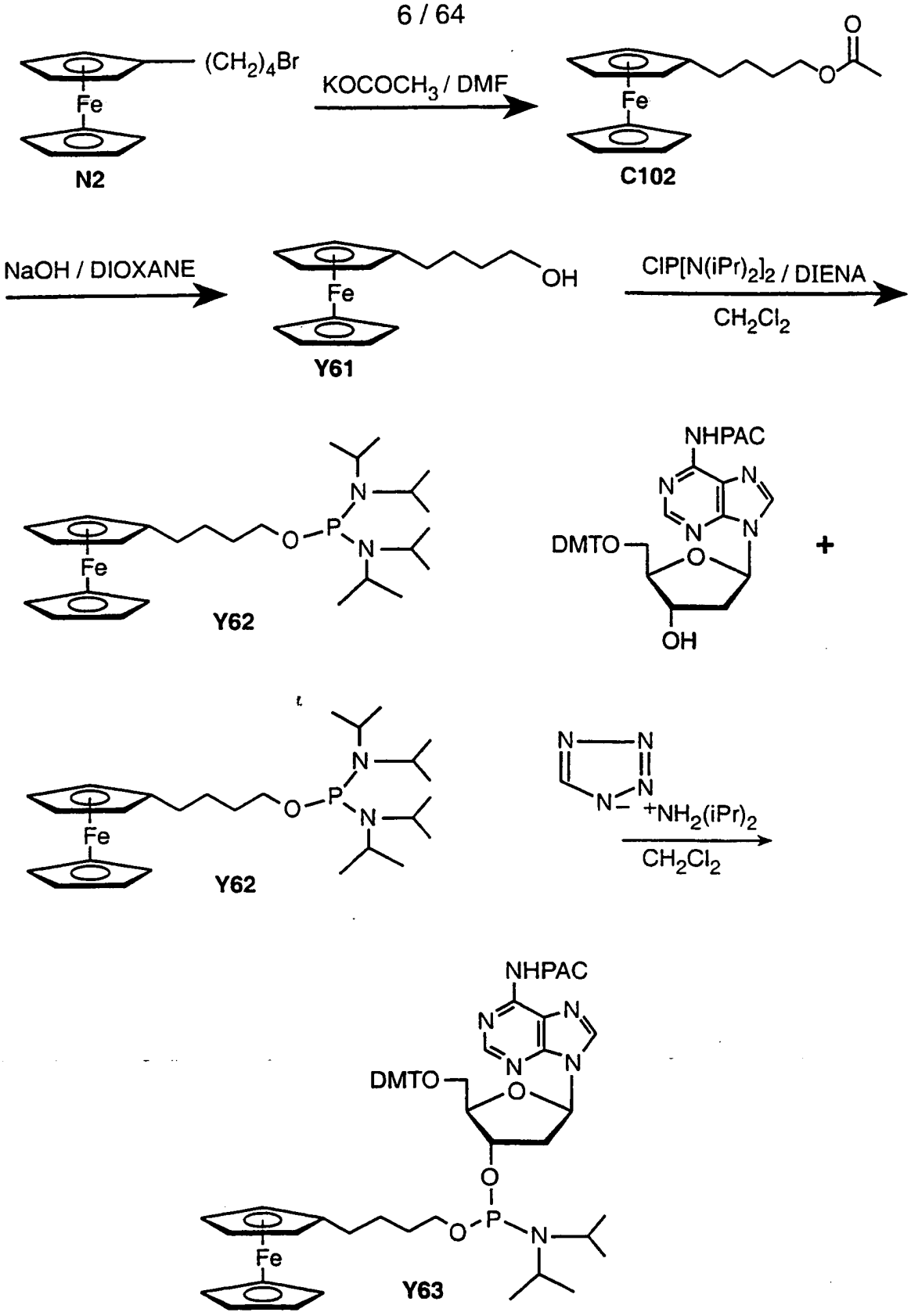

(CH2)4Br
Fe
N2
KOCOCH3 / DMF
O
O
Fe
C102
NaOH / DIOXANE
OH
Fe
Y61
ClP[N(iPr)2]2 / DIENA
CH2Cl2
N
O
P
N
Fe
Y62
NHPAC
N
N
N
N
DMTO
O
OH
+
Y62
N
N
N
N
+NH2(iPr)2
CH2Cl2
NHPAC
N
N
N
N
DMTO
O
O
P
O
N
Fe
Y63

FIG._4

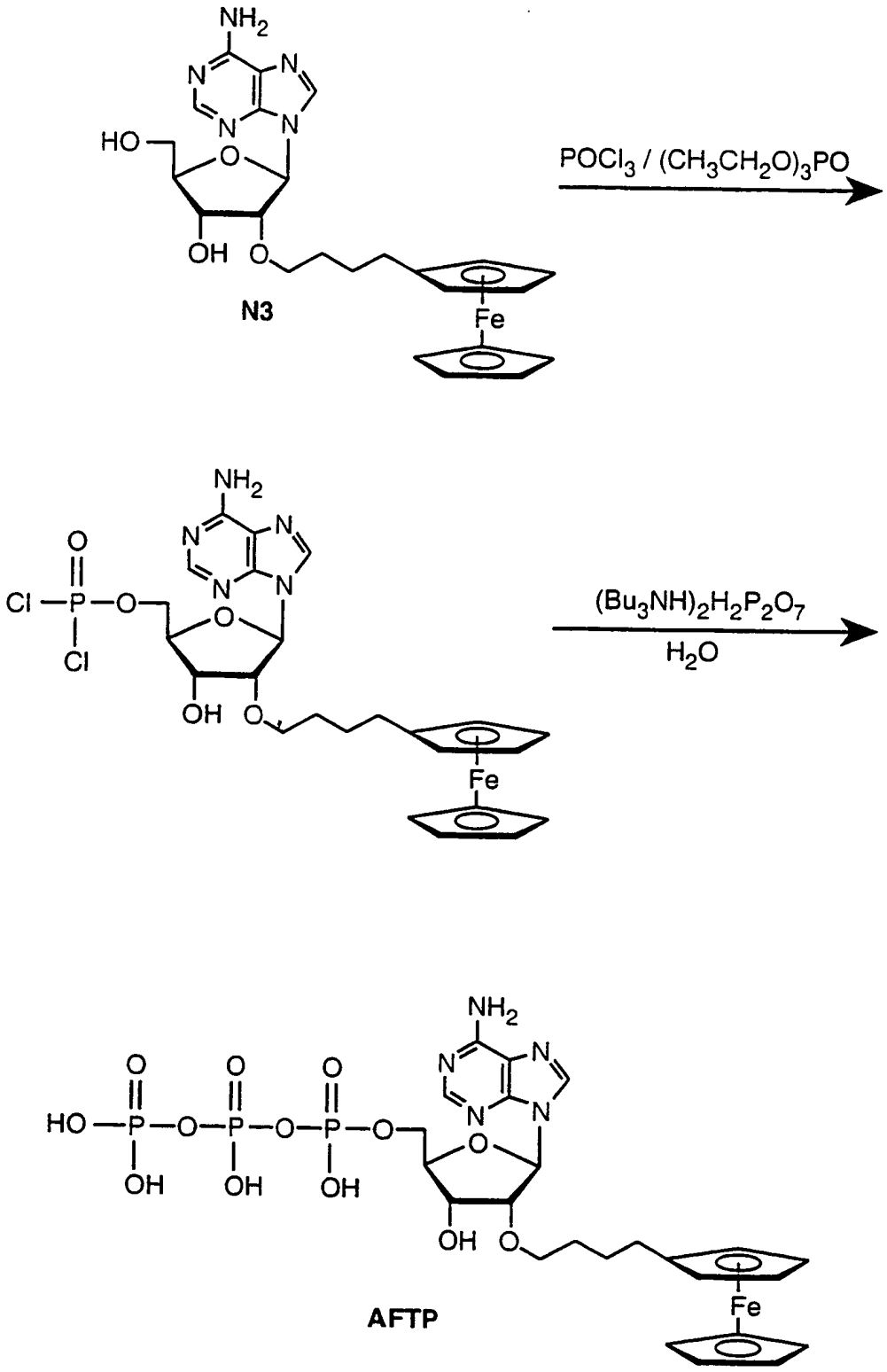


FIG._5

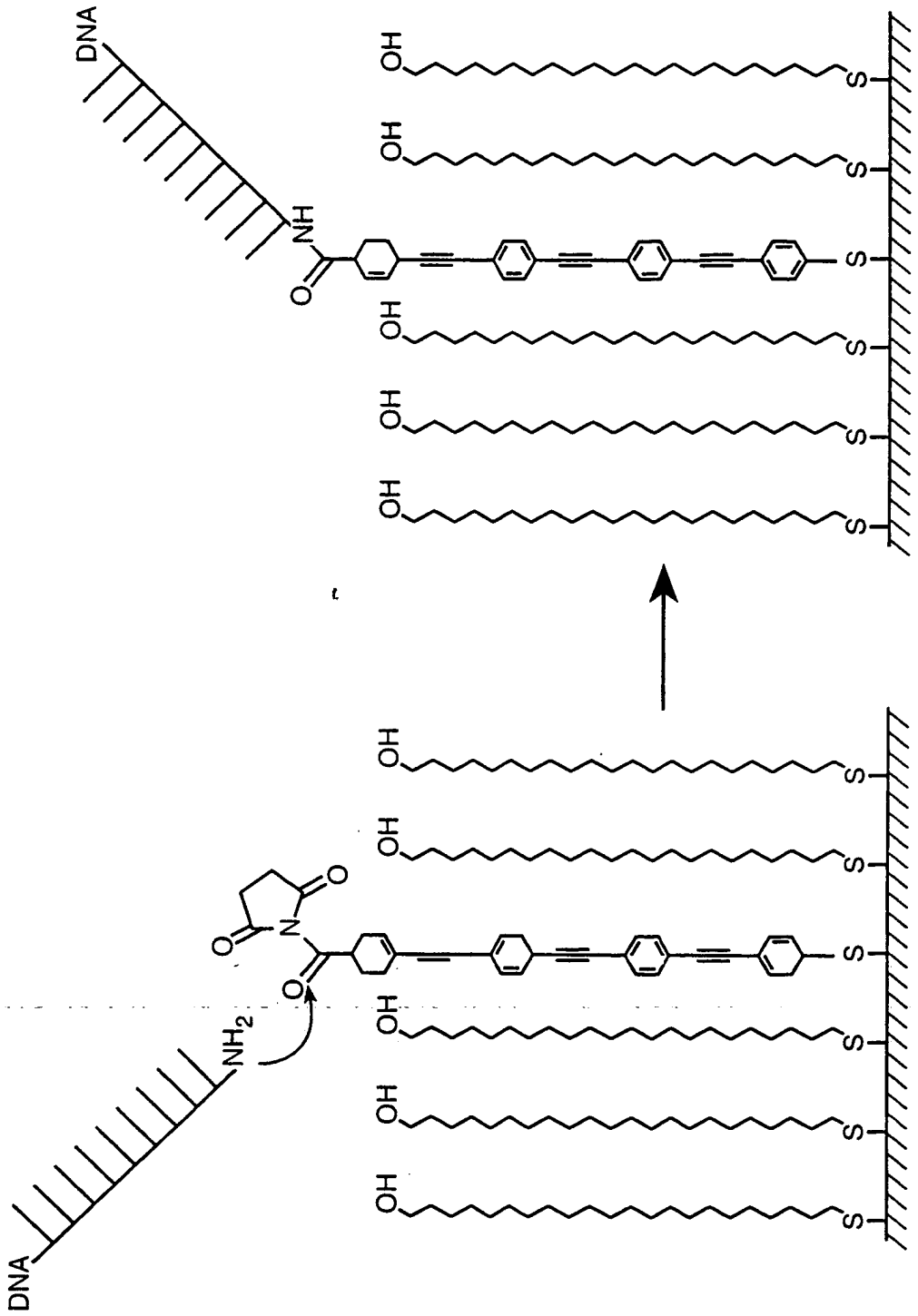


FIG._6

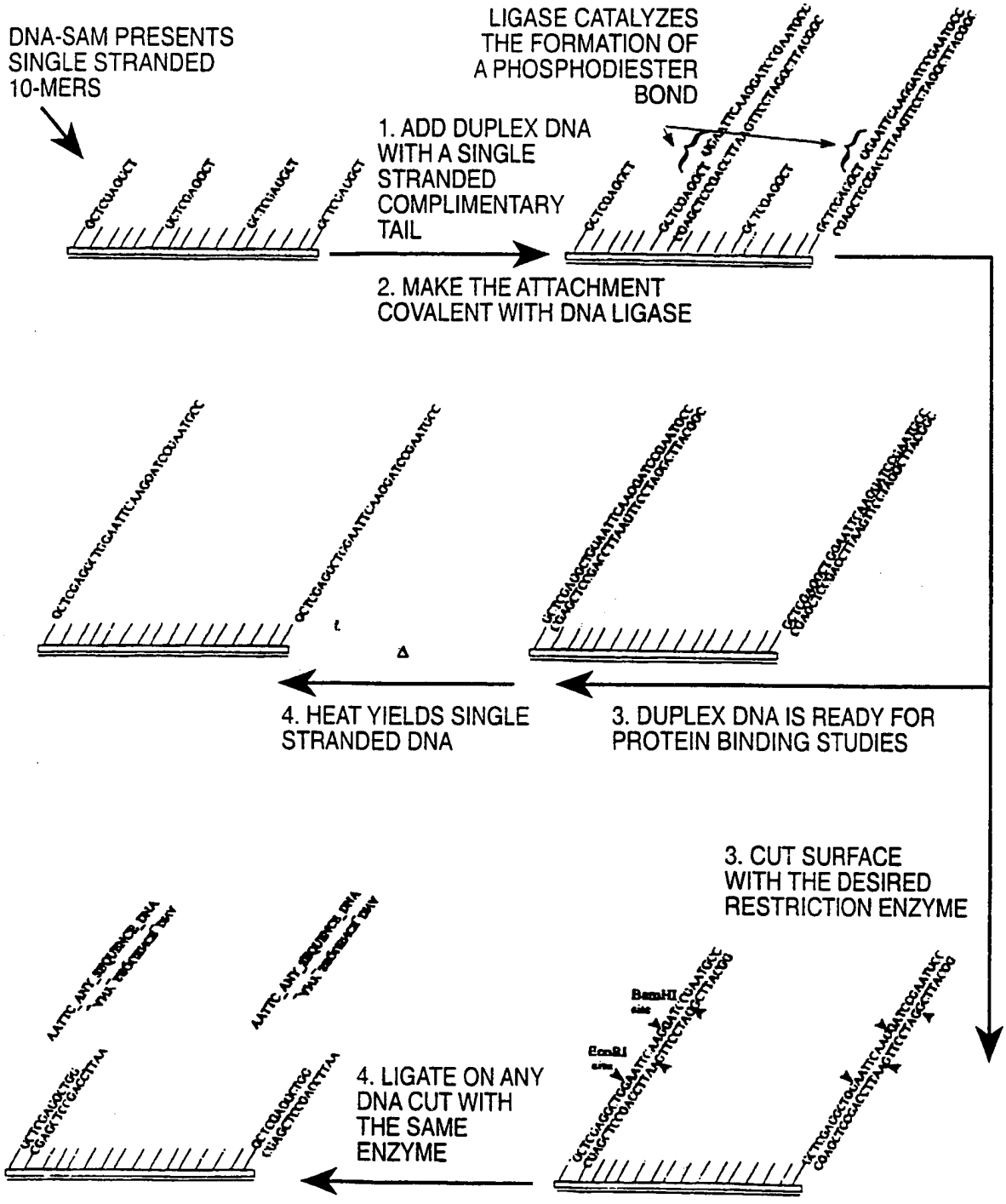
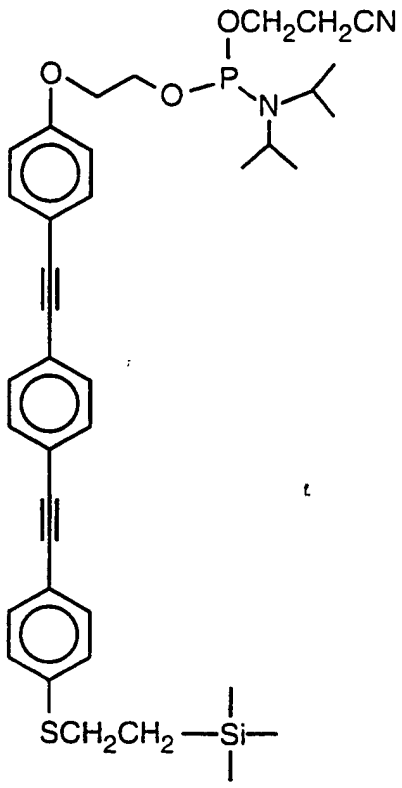
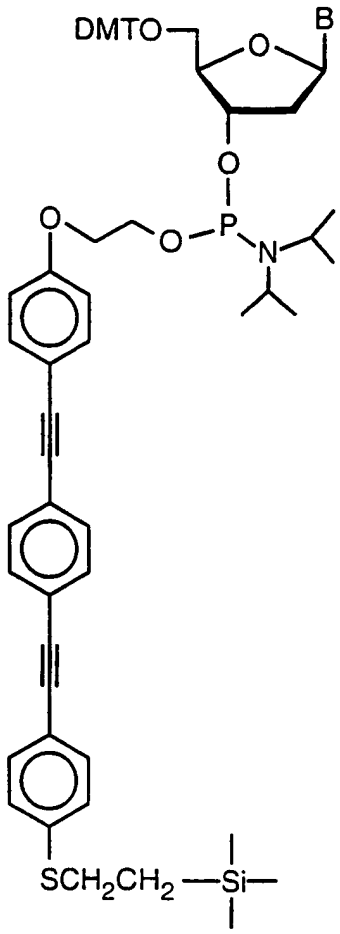


FIG._7



5' - ATTACHMENT

FIG._8A



ANY POSITION ATTACHMENT

FIG._8B

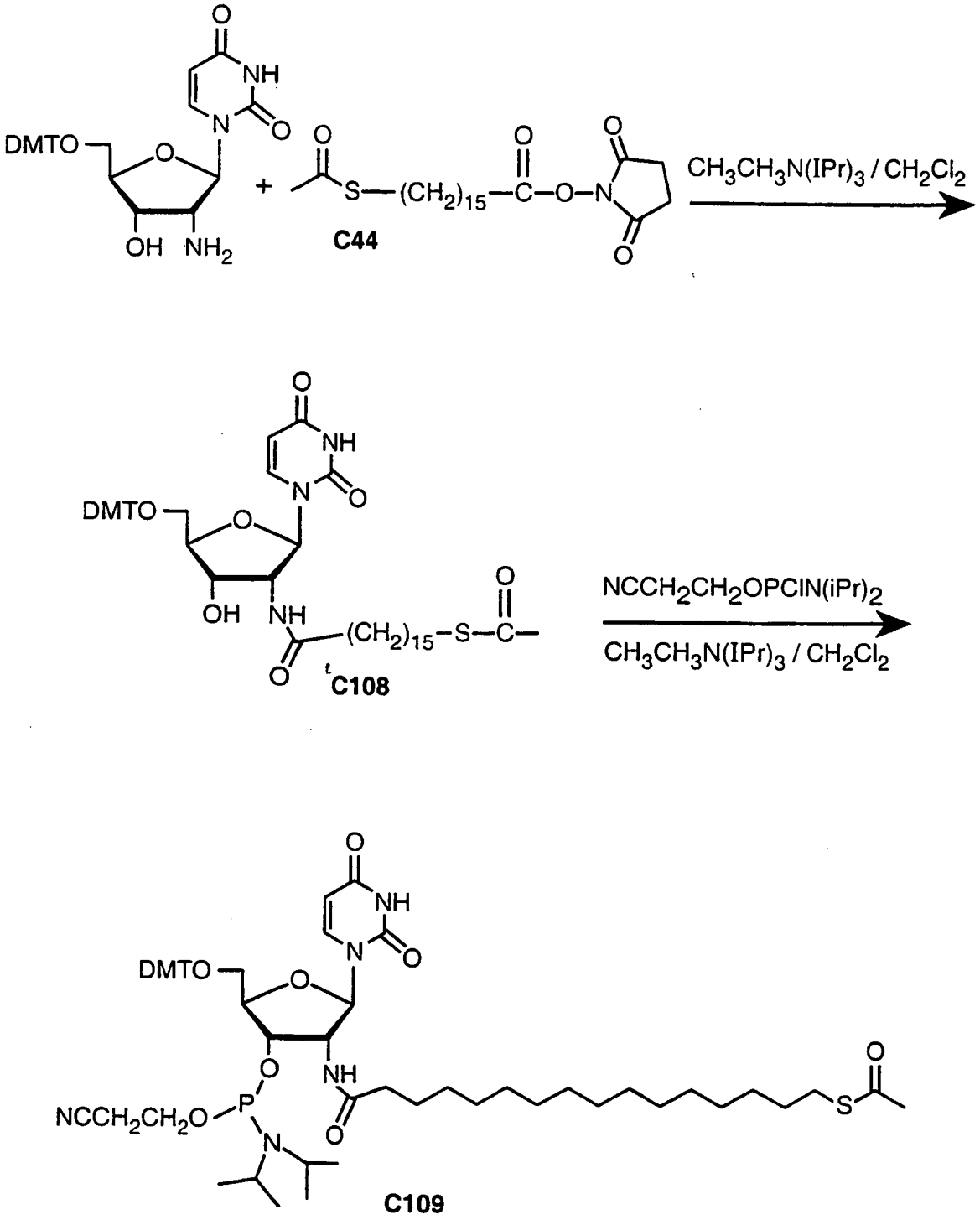


FIG._9

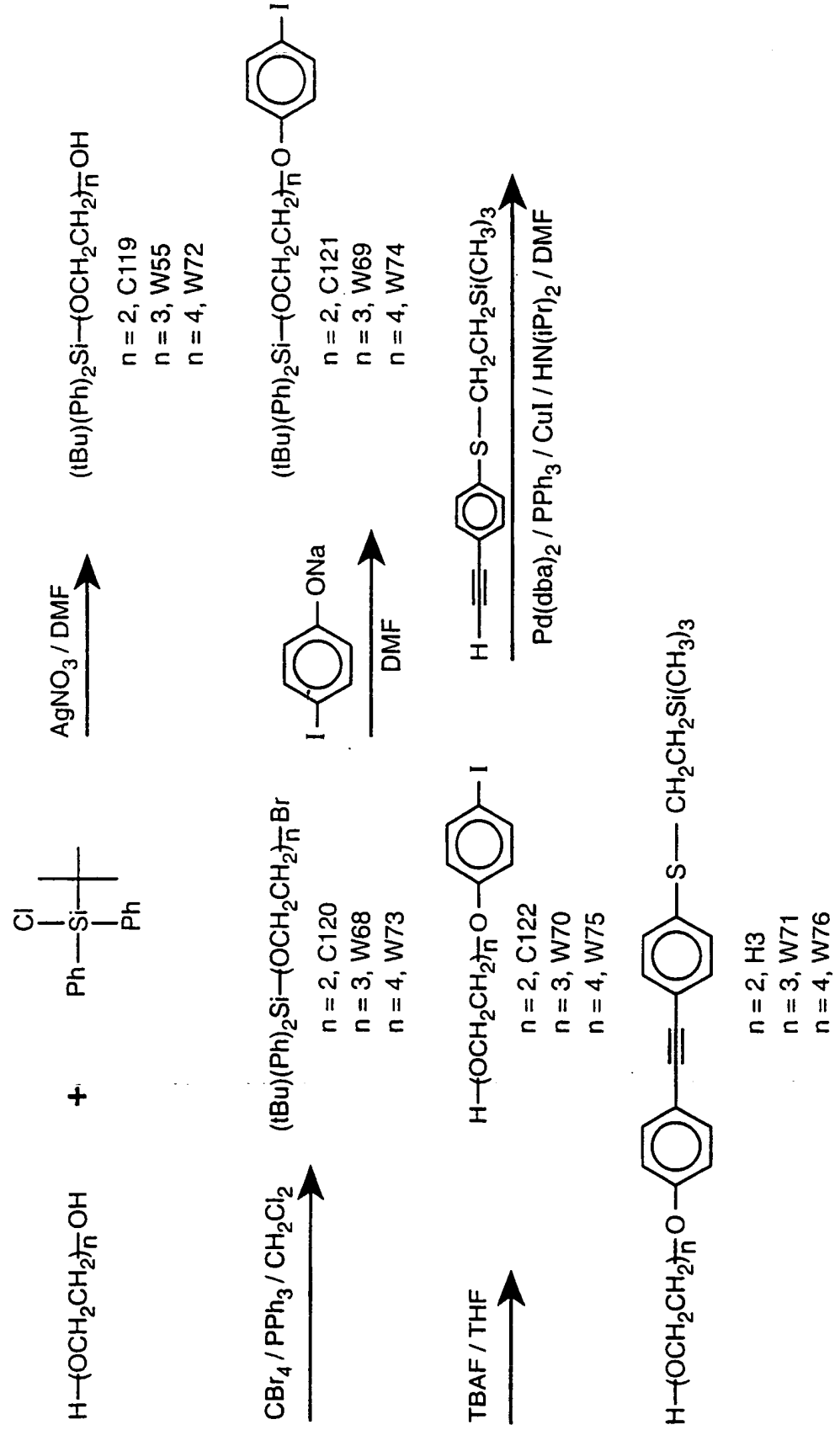


FIG._10

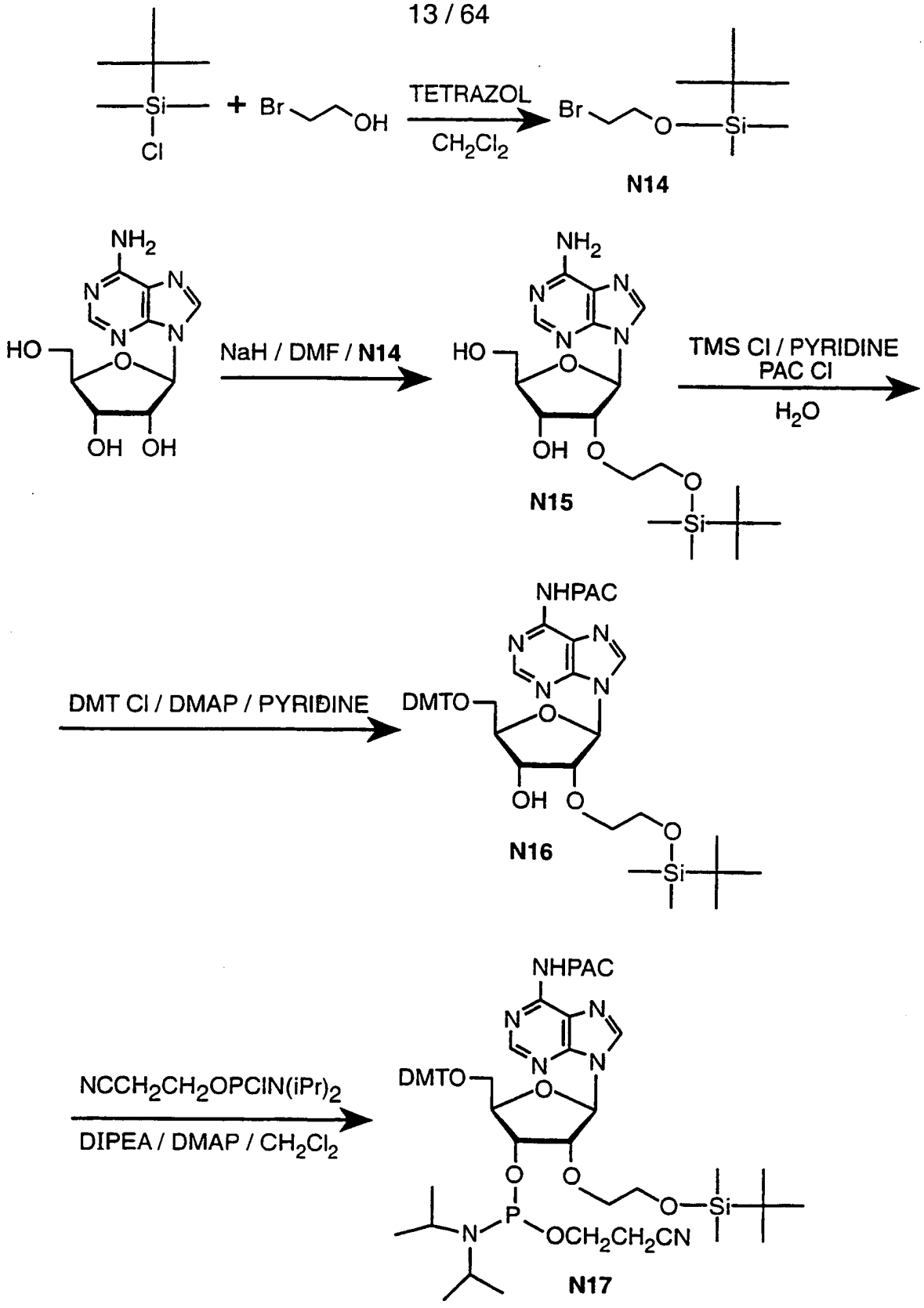

TETRAZOL
CH_2Cl_2
N14
NaH / DMF / N14
N15
TMS Cl / PYRIDINE
PAC Cl
H_2O
DMT Cl / DMAP / PYRIDINE
N16
$NCCH_2CH_2OPCIN(iPr)_2$
DIPEA / DMAP / CH_2Cl_2
N17

FIG._11A

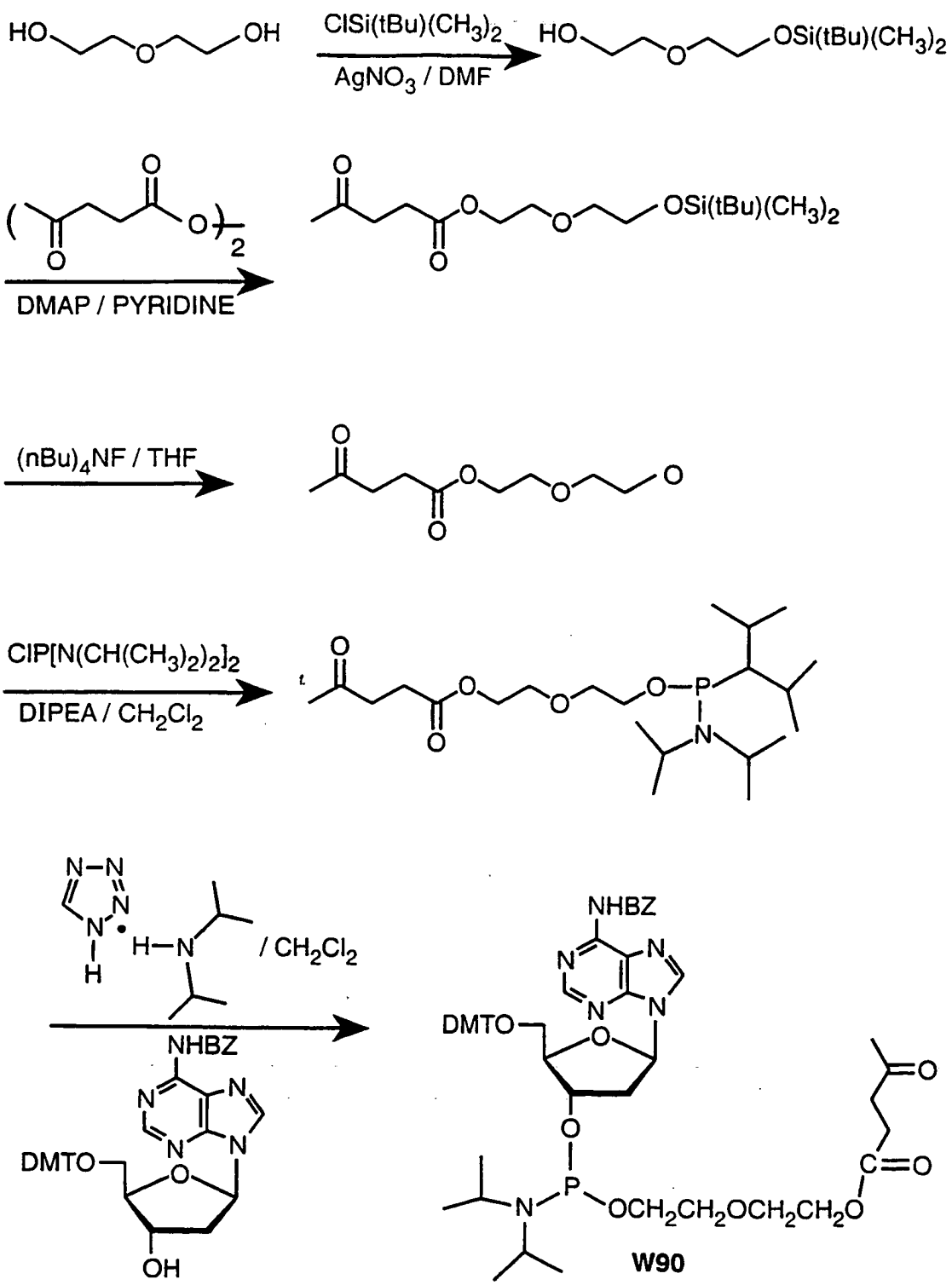


FIG._11B

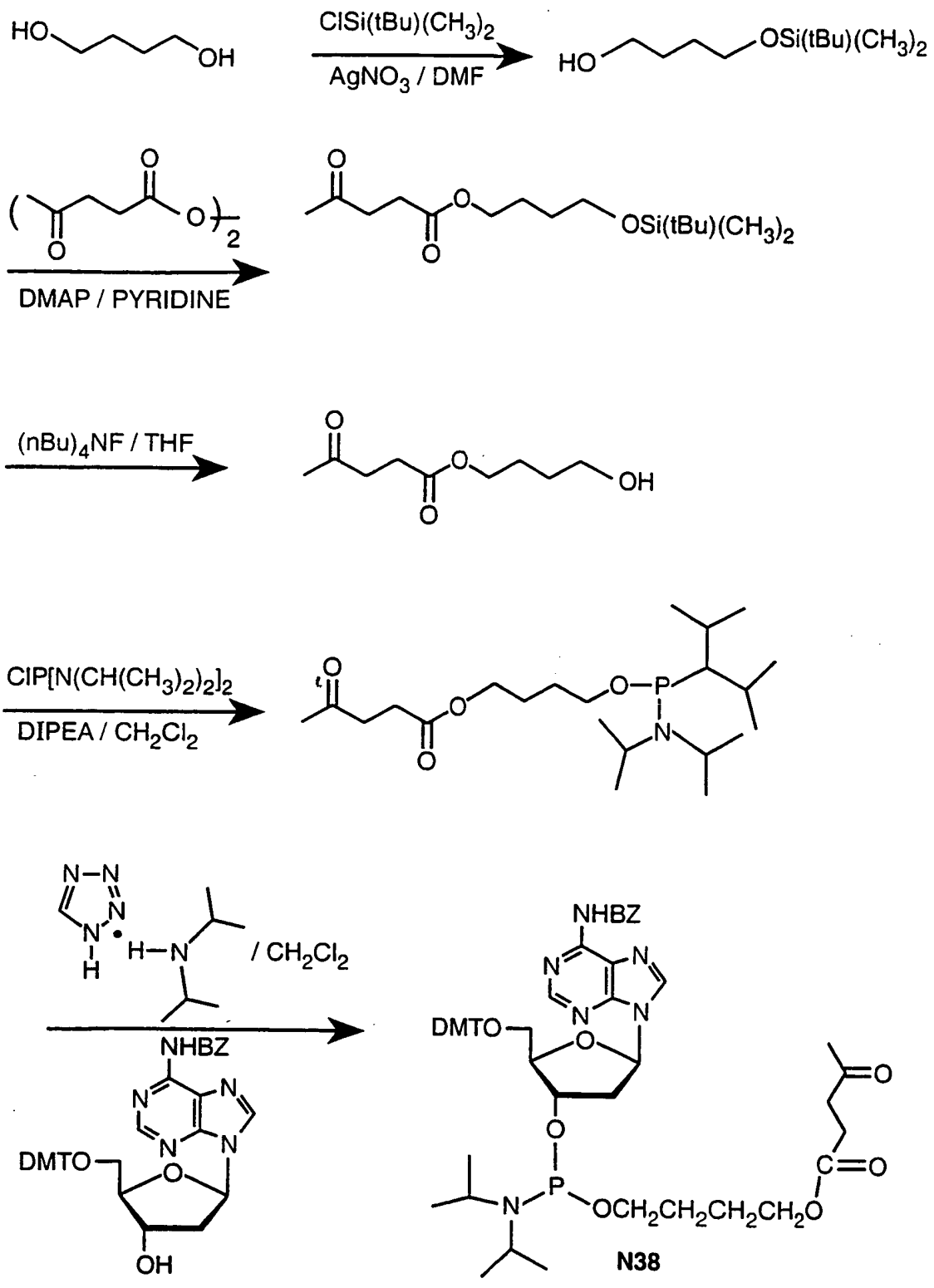


FIG._11C

FIG._12

FIG._12A FIG._12B FIG._12C

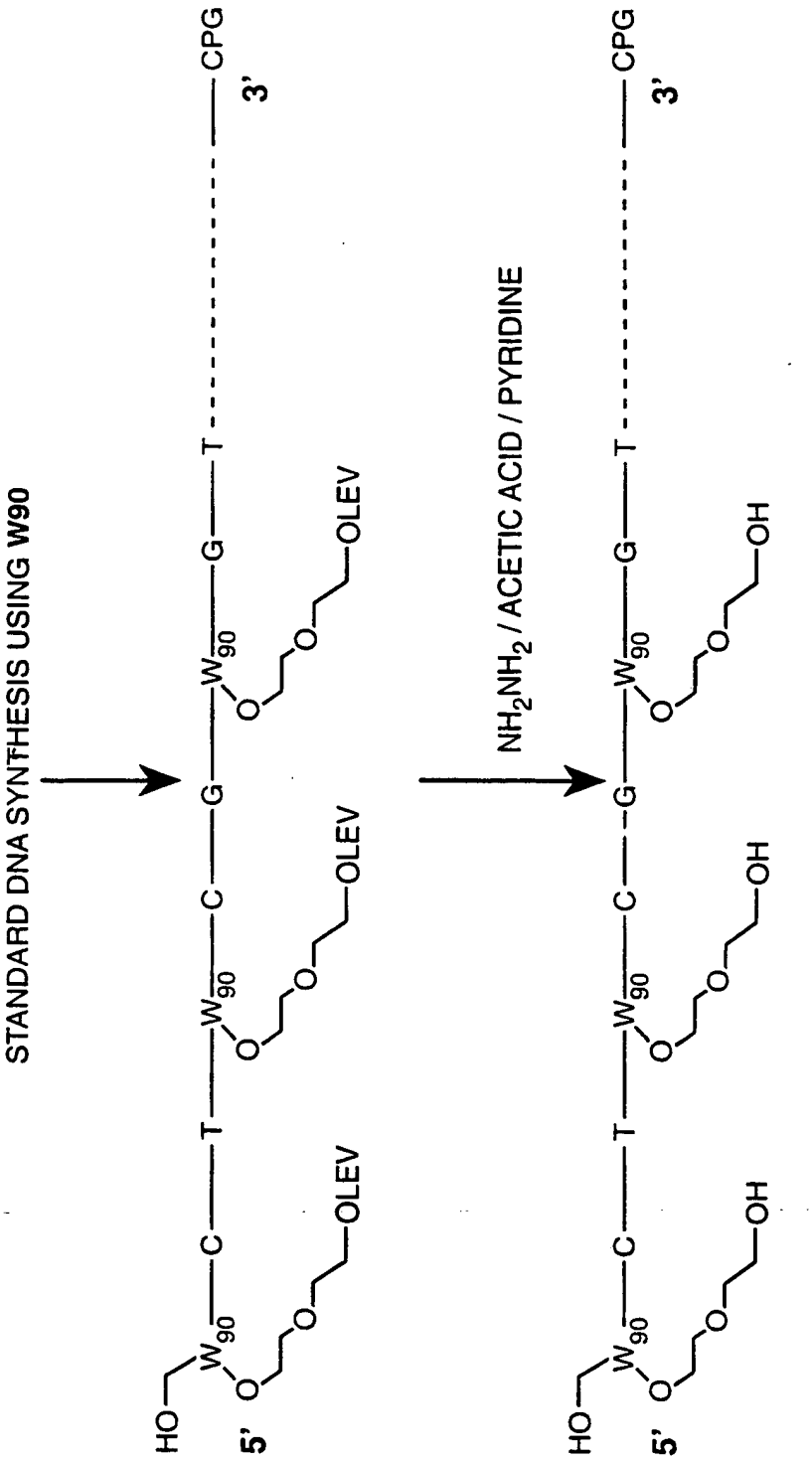


FIG._12A

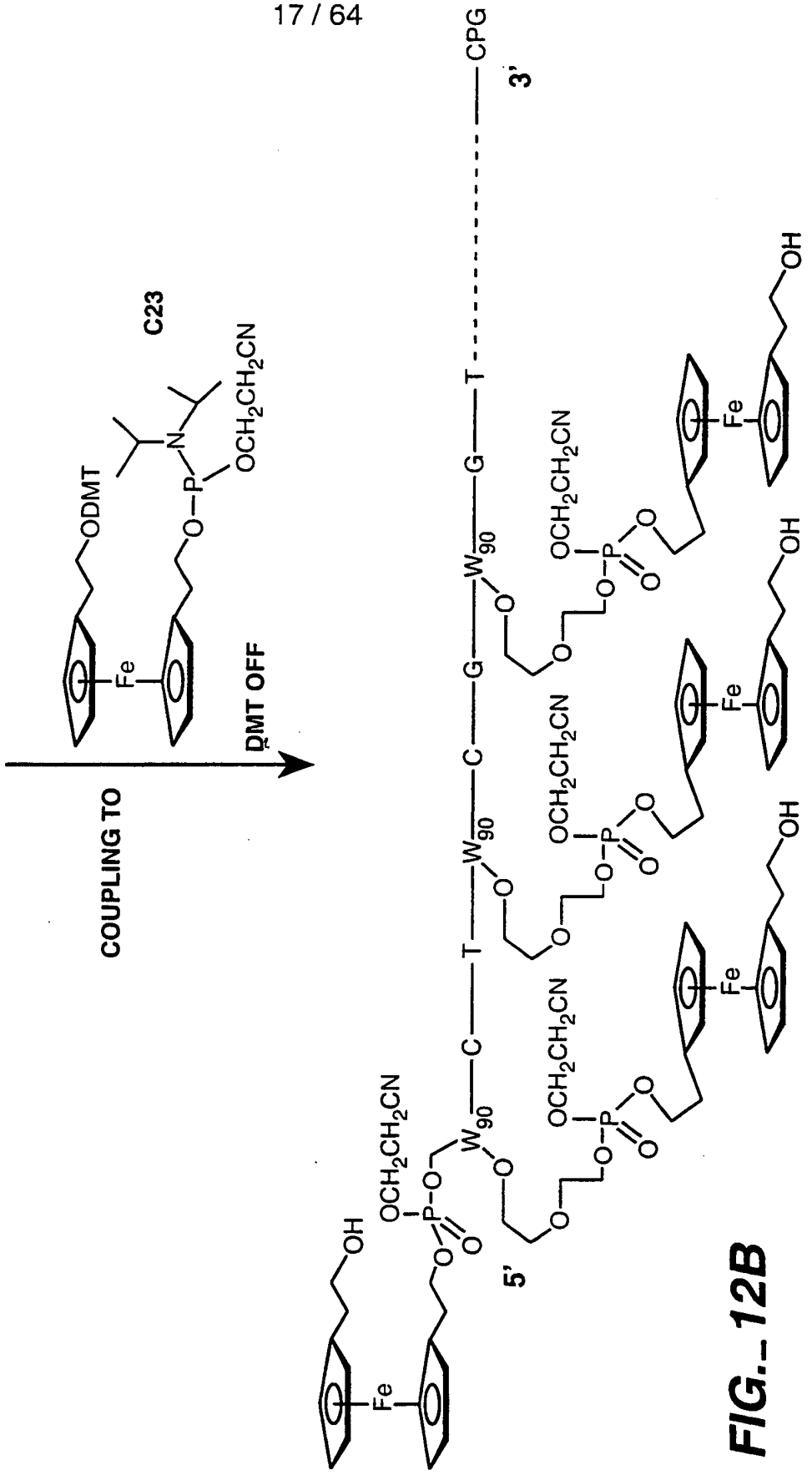


FIG._12B

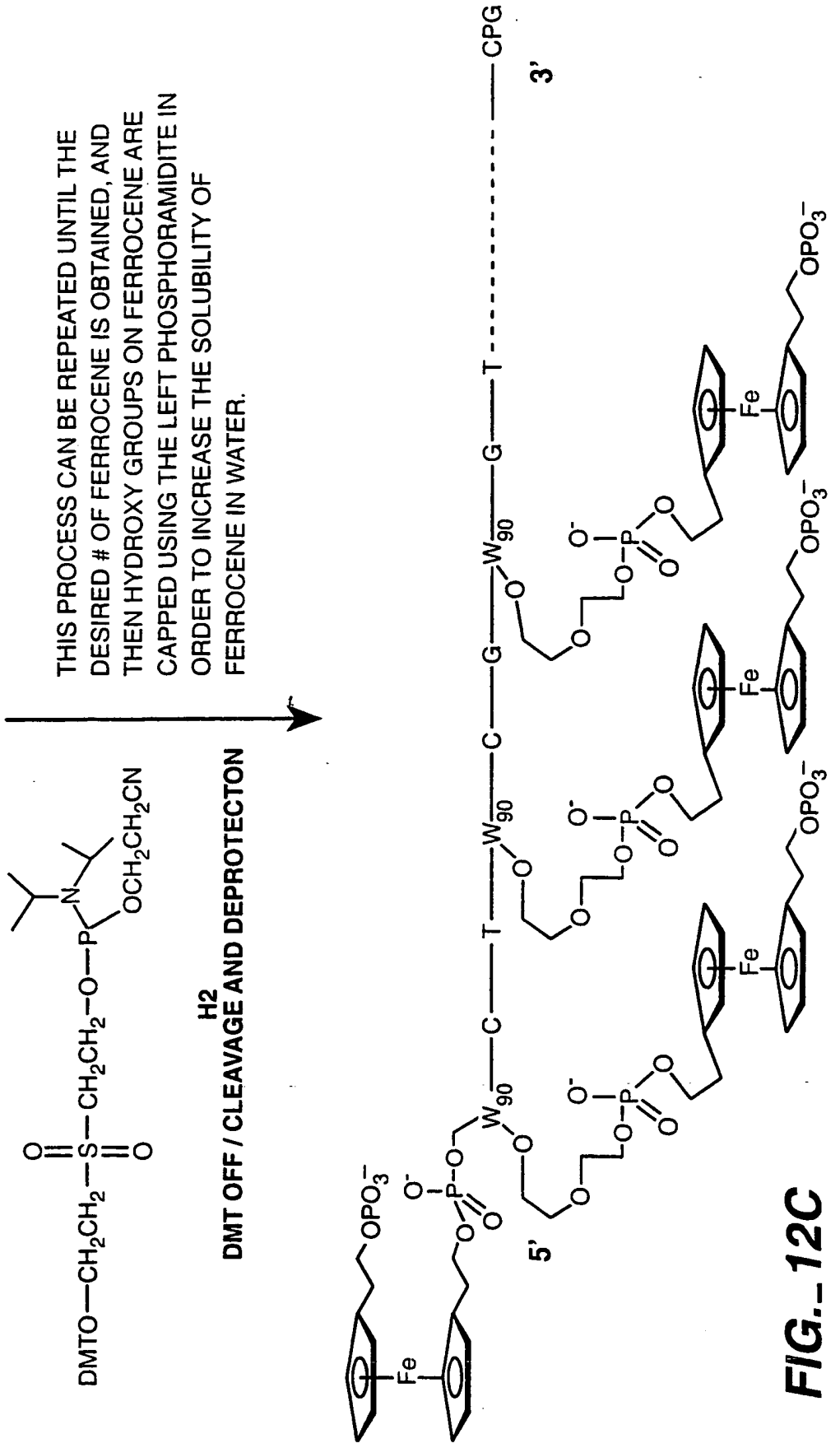


FIG._12C

FIG._13

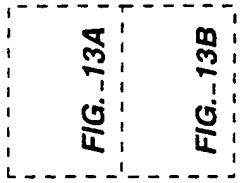
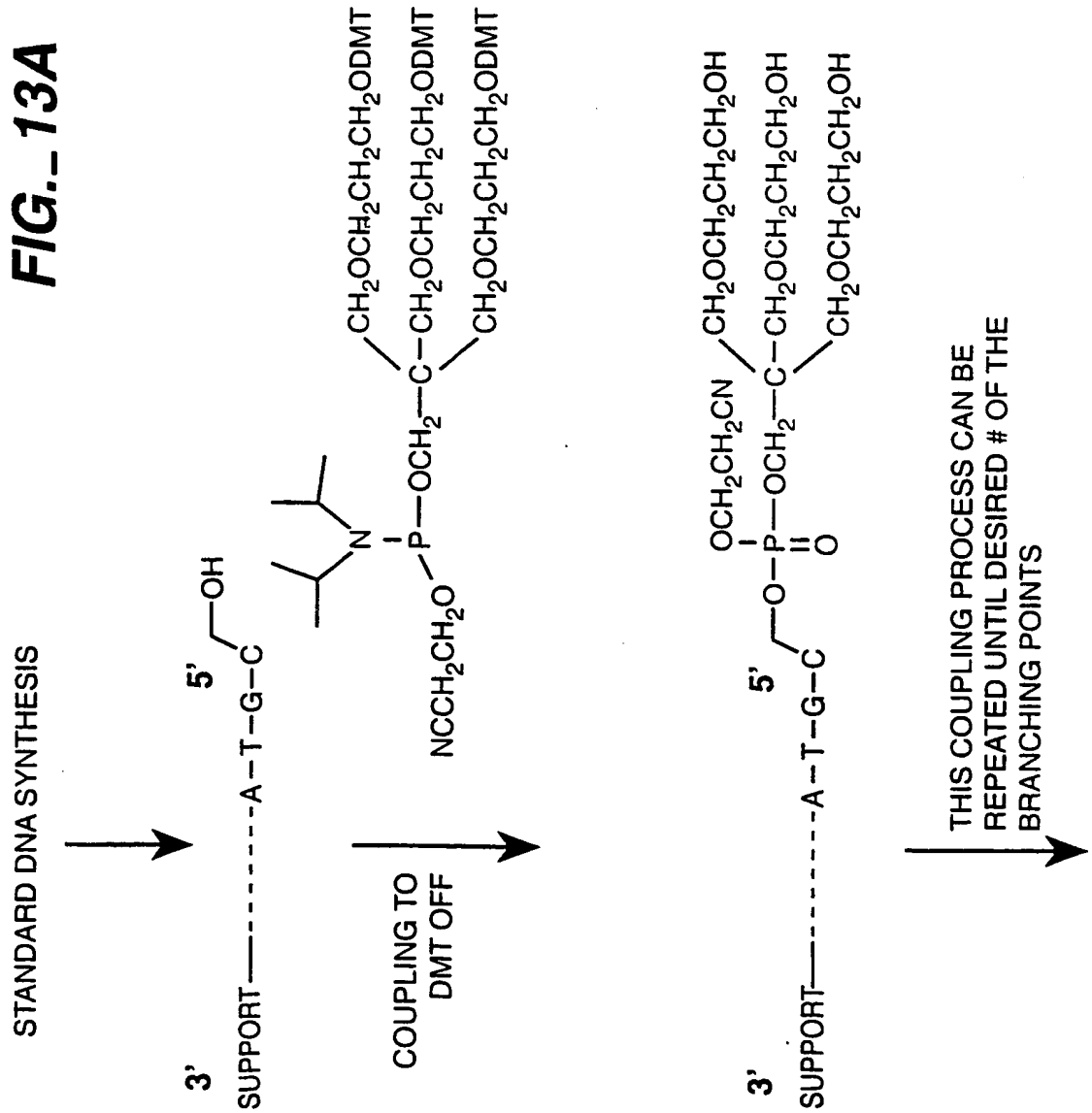


FIG._13A



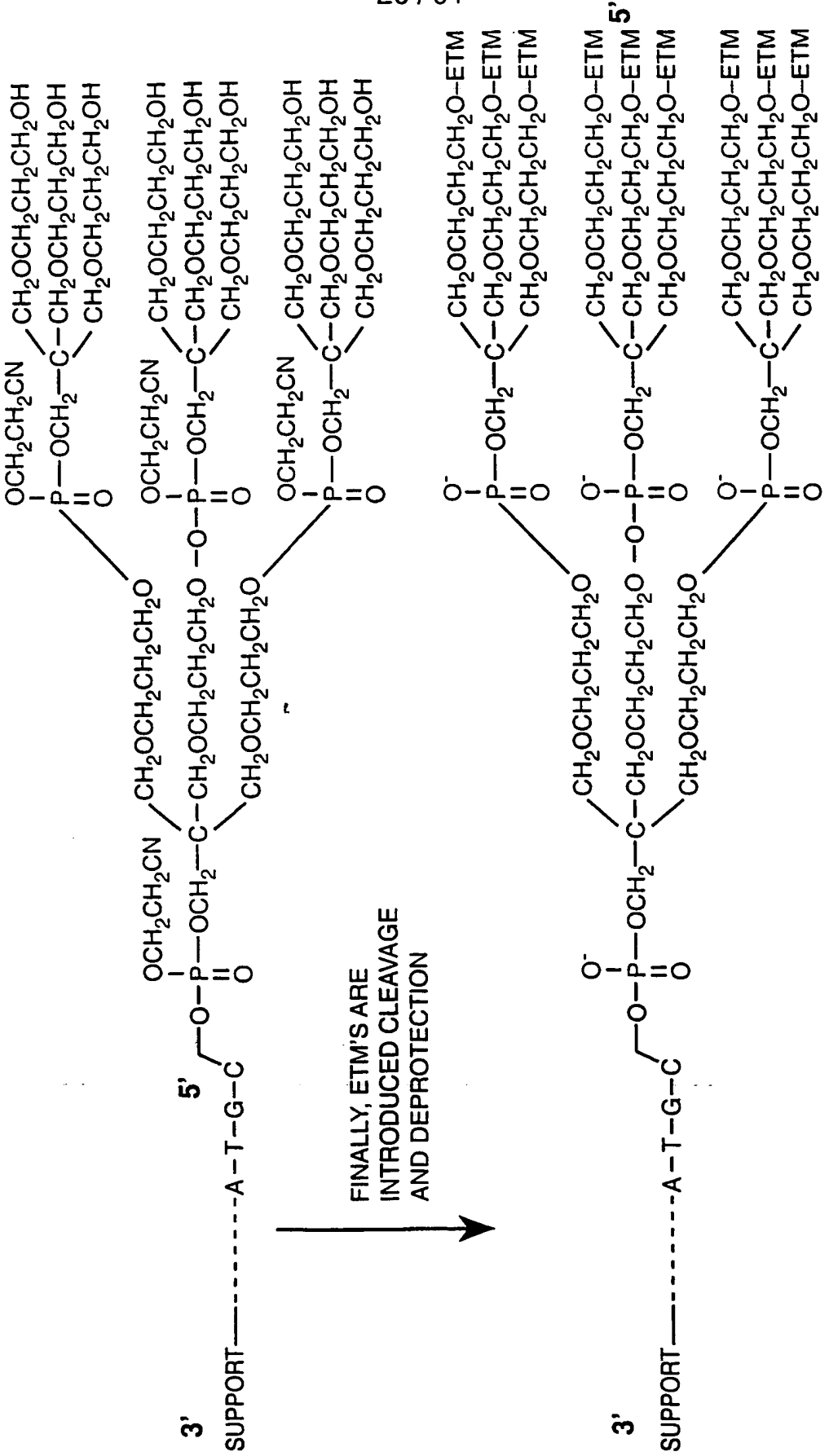

FINALLY, ETM'S ARE
INTRODUCED CLEAVAGE
AND DEPROTECTION
3'
5'
SUPPORT
A T G C
OCH2CH2CN
CH2OCH2CH2CH2OH
CH2OCH2CH2CH2O
O-ETM
O-
P
O
OCH2
C

FIG._13B

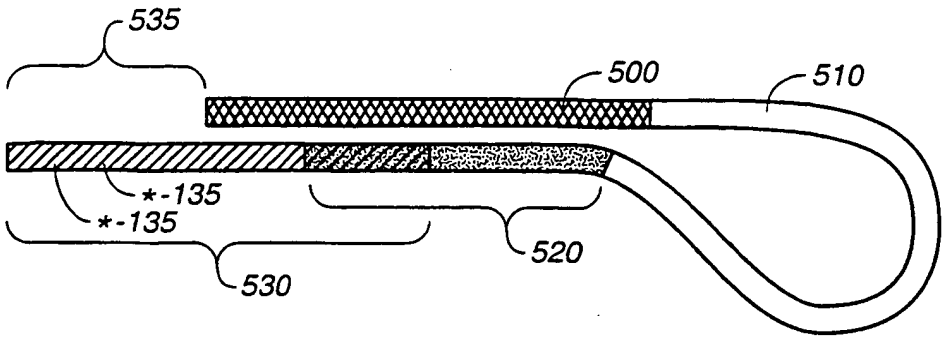


FIG._14

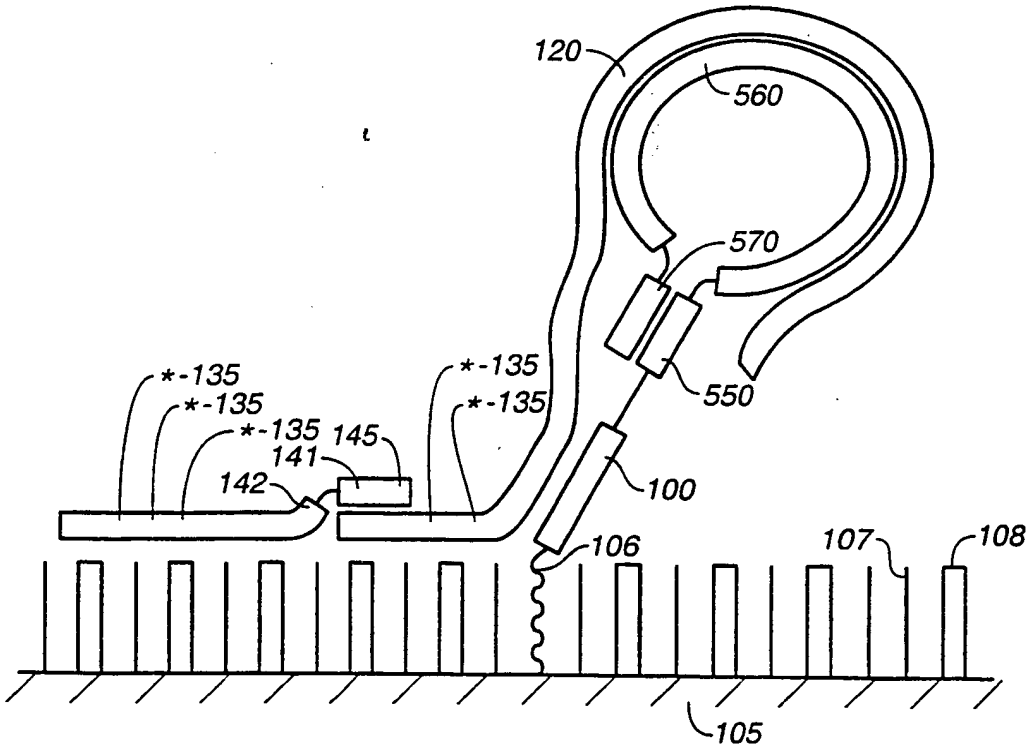


FIG._18

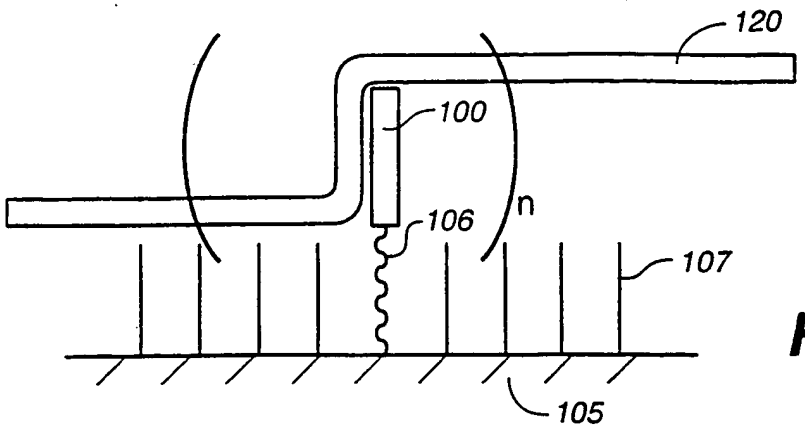


FIG._15A

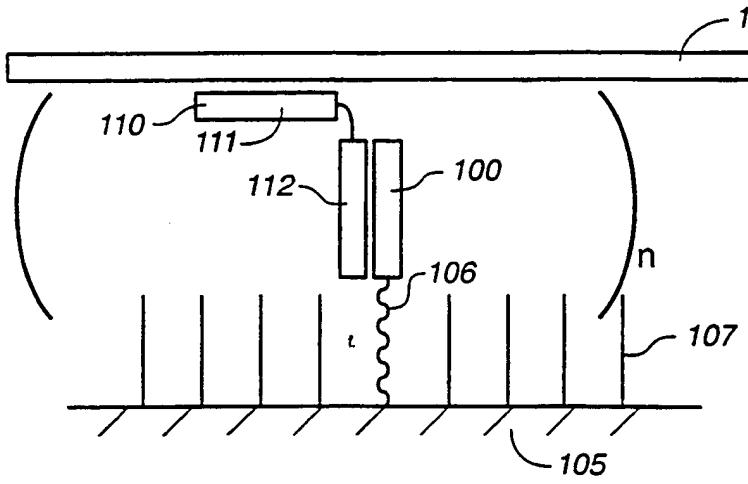


FIG._15B

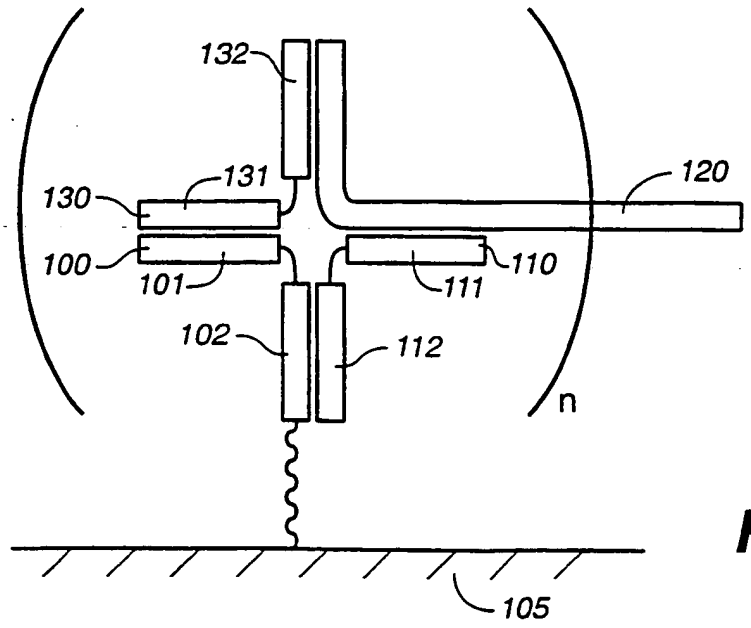


FIG._15C

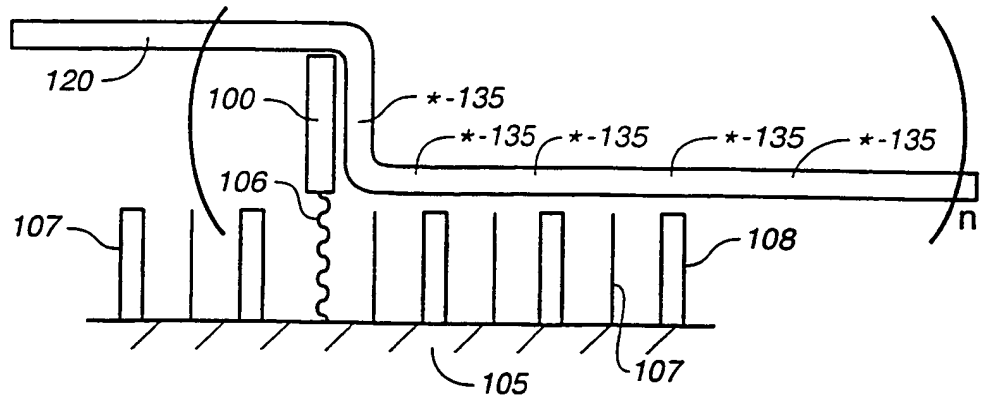


FIG._16A

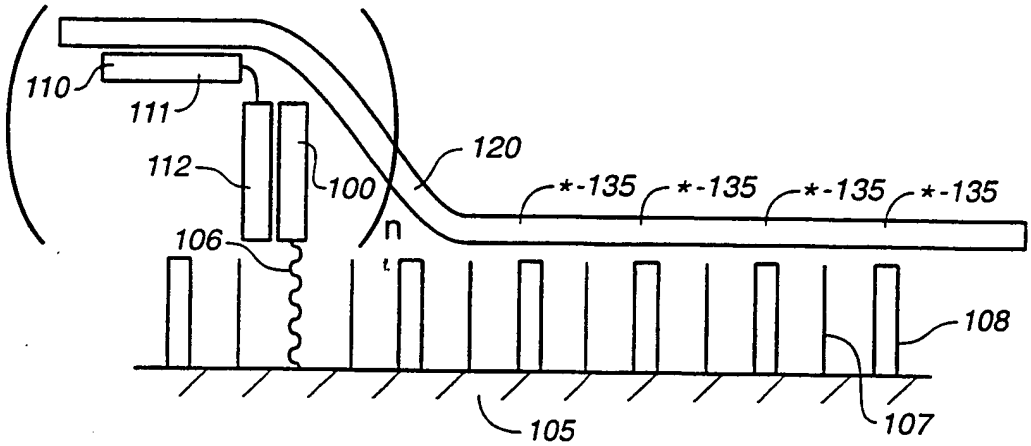


FIG._16B

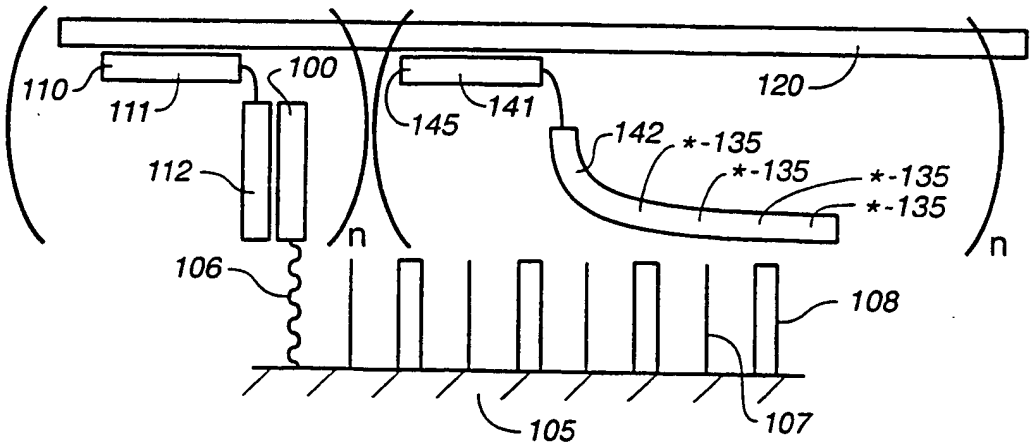


FIG._16C

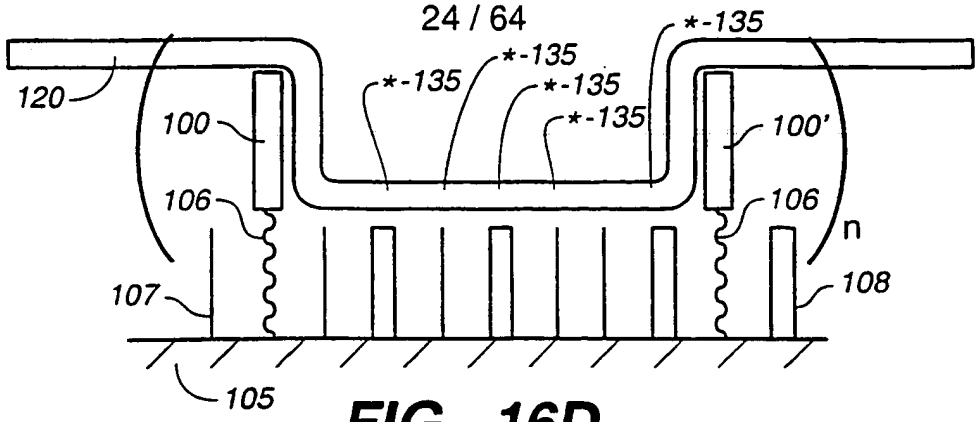


FIG._16D

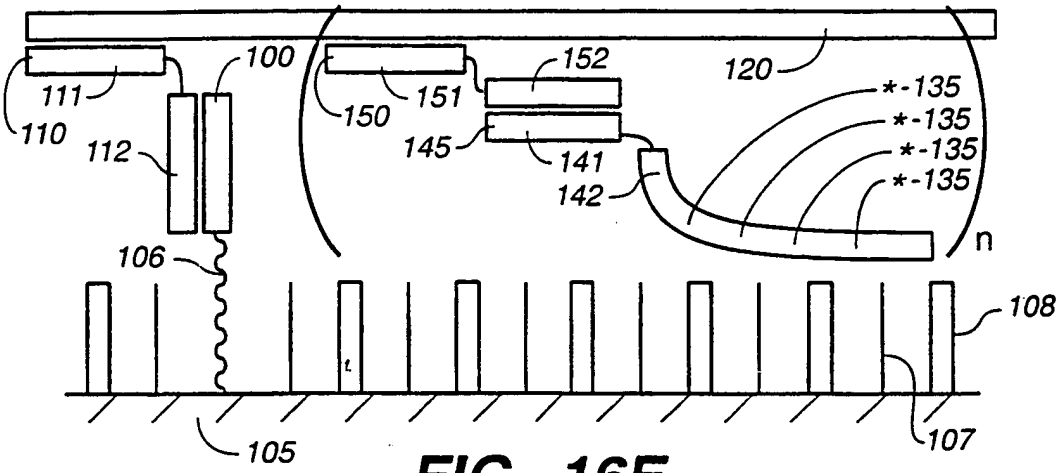


FIG._16E

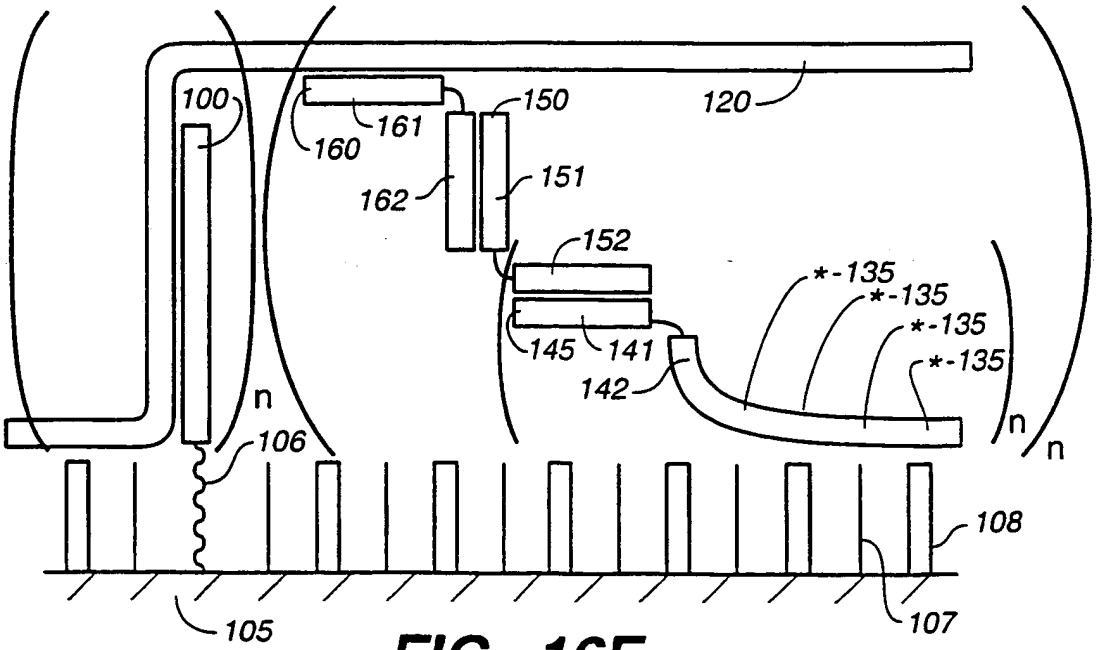


FIG._16F

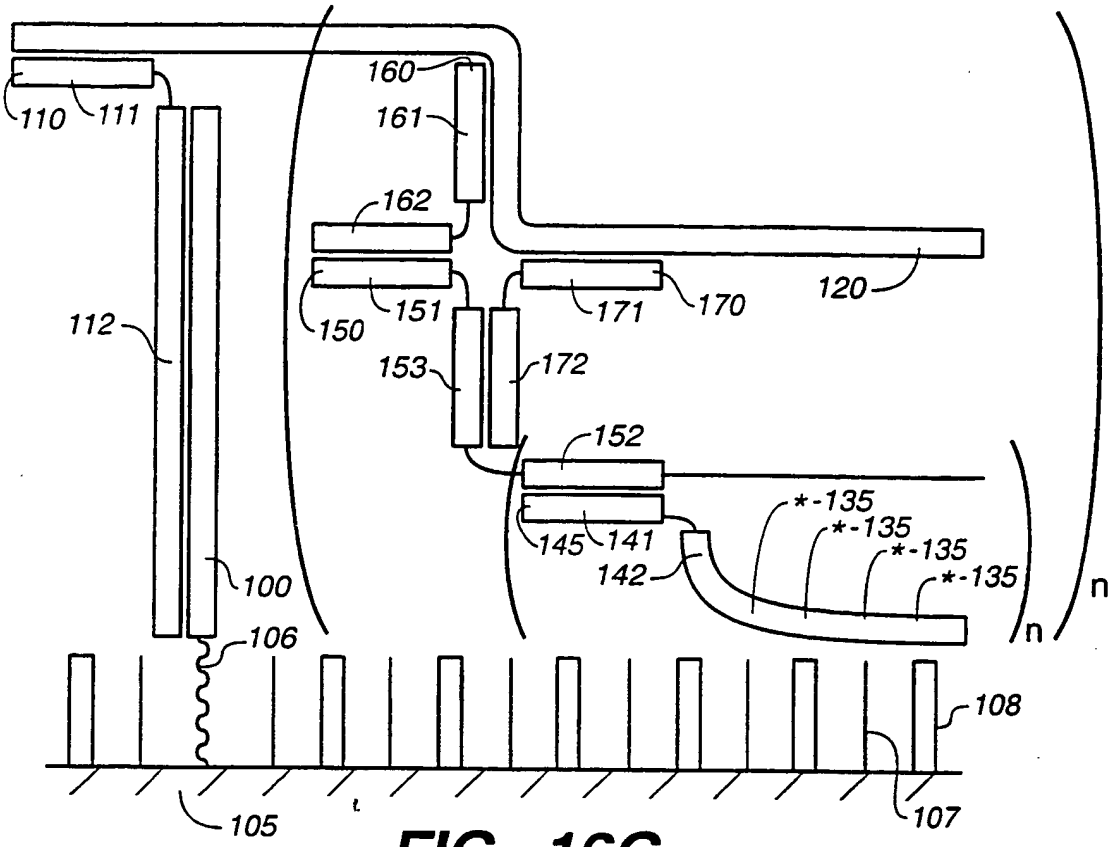


FIG._16G

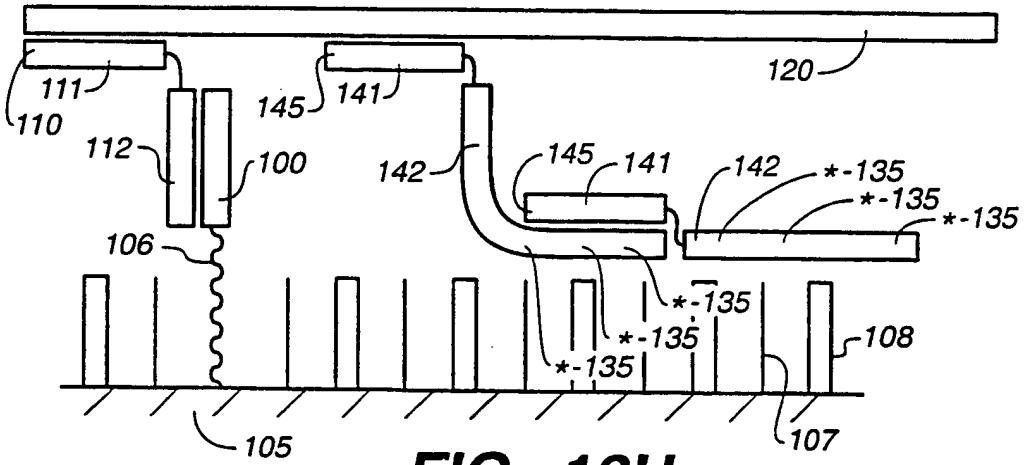
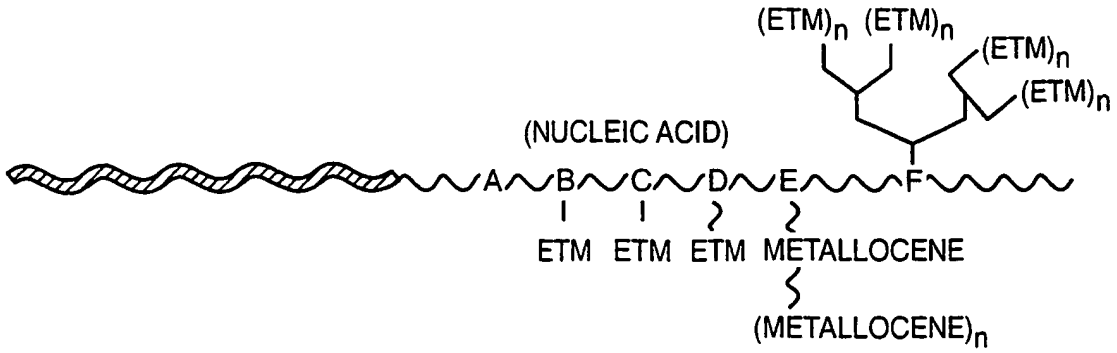


FIG._16H

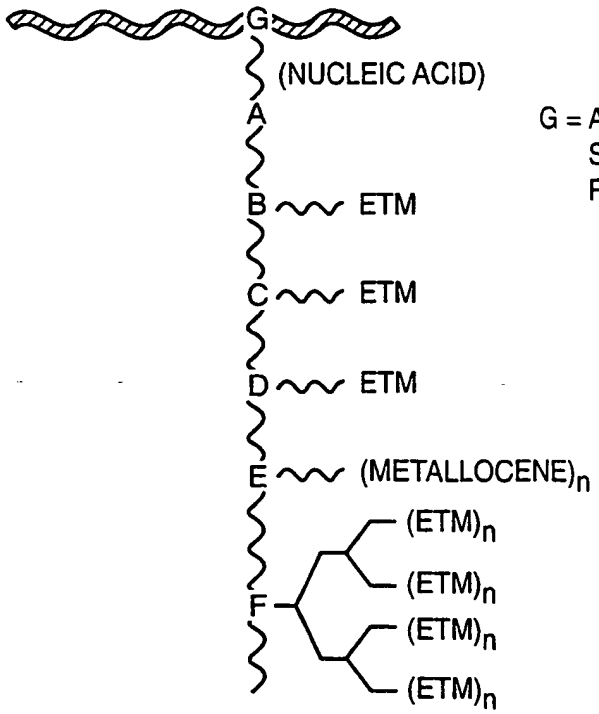
= FIRST HYBRIDIZABLE PORTION OF LABEL PROBE

= RECRUITMENT LINKER



A = NUCLEOSIDE REPLACEMENT
B = ATTACHMENT TO A BASE
C = ATTACHEMENT TO A RIBOSE
D = ATTACHMENT TO A PHOSPHATE
E = METALLOCENE POLYMER, ATTACHED TO A RIBOSE, PHOSPHATE, OR BASE
F = DENDRIMER STRUCTURE, ATTACHED VIA A RIBOSE, PHOSPHATE OR BASE

FIG._17A



G = ATTACHMENT VIA A "BRANCHING STRUCTURE", THROUGH RIBOSE, PHOSPHATE OR BASE

FIG._17B

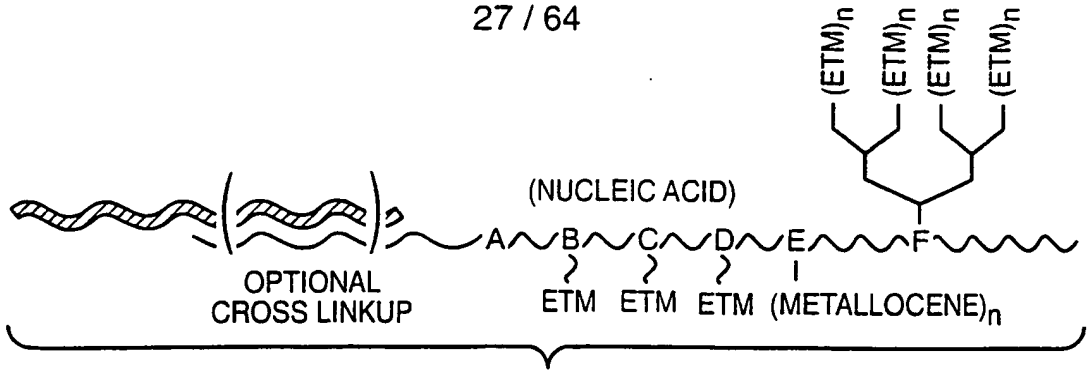


FIG._17C

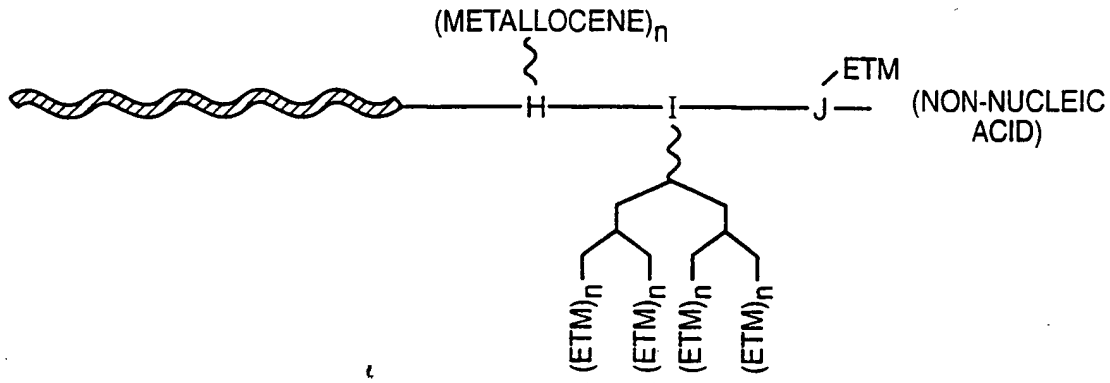


FIG._17D

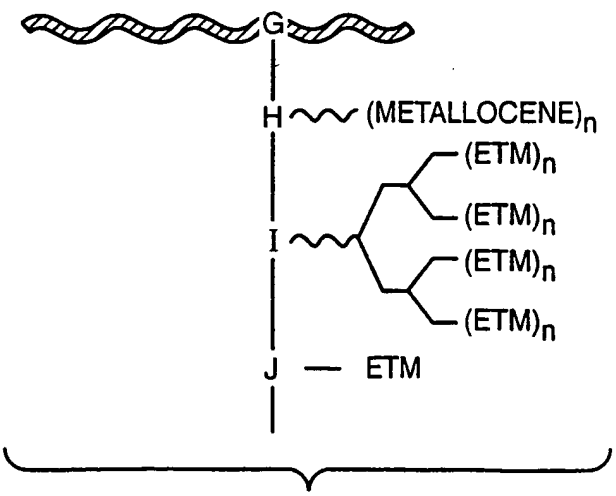


FIG._17E

FIG._19

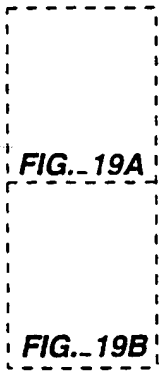


FIG._19A

D179
5' – A(C15)CCTGGTCTTGACATCCACGGAAGGCGTGGAAATACGTATTCGTGCCTA – 3'

D309 (Dendrimer)
5' – (W38)(Branching)(Branching)CATGGTTAACGTCAATTGCTGCGGTTATTAA – 3'

D295
5' - (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)CCCATGGTTAGACTGAATTGCTGCGGTTATTAA – 3'

D297
5' - (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)TATGCTCTTGATGGTGCTGTGGAAATCTACTGG – 3'

D298
5' – (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)ATGGTGCTGTGGAAATCTACTGG – 3'

D296
5' - (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)TGACTGAATTGCTGCGGTTATTAA – 3'

D112
5' – CTTCCGTGGATGTCAAGACCAGGAU – 4 unit wire (C11) – 3'

D94
5' – ACCATGGACACAGAU – 4 unit wire (C11) – 3'

D109
5' – CTGCGGTTATTAACU – 4 unit wire (C11) – 3'

2Tar
5' – TAG GCA CGA ATA CGT ATT TCC ACG ATA AAT ATA ATT AAT AAC CGC AGC AAT TGA CGT ATA AAG CTA TCC CAG TAG ATT TCC ACA GC – 3'

D349
5' – A(C15)C (C15)GT GTC CAT GGT AGT AGC TTA TCG TGG AAA TAC GTA TTC GTG CCT A – 3'

D382
5' – (Y63)G(Y63) CT(Y63) C(Y63)G (Y63)C(Y63) CCC ATG GTT AGA CTG AAT TGC TGC GGT TAT TAA – 3'

D383
5' – (Y63)G(Y63) CT(Y63) C(Y63)G (Y63)C(Y63) CCC ATG GTT AGA CTG GCT GTG GAA ATC TAC TGG –3'

D468
5' – (N6)G(N6) CT(N6) C(N6)G (N6)C(N6) (glen)(glen)(glen) CTT TAC TCC CTT CCT CCC CGC TGA AAG TAC – 3'

D449
5' – CGG AGT TAG CCG GTG CTT CTT CTG CGG G(C131)(C131) (C131)(C131)(N6) G(N6)C T(N6)C (N6)G(N6) C(N6)T – 3'

D417
5' – CTT TAC TCC CTT CCT CCC CGC TGA AAG TAC TTT ACA ACC C – 3'

EU1
5' – ATC CTG GTC TTG ACA TCC ACG GAA GAT GTC CCT ACA GTC TCC ATC AGG CAG TTT CCC AGA CA - 3'

MT1
5' – TCT ACA TGC CGT ACA TAC GGA ACG TAC GGA GCA TCC TGG TCT TGA CAT CCA CGG AAG – 3'

D358
5' – (N6)G(N6) CT(N6) C(N6)G (N6)C(N6) CCG TAT GTA CGG CAT GTA GA – 3'

D334
5' – GCT ACT ACC ATG GAC ACA GAU – 4 unit wire (C11) – 3'

D335
5' – ACA GAC ATC AGA GTA ATC (N6)GC C(N6)G TC(N6) TGG (N6)T – 3'

LP280
5' – GAT TAC TCT GAT GTC TGT CCA TCT GTG TCC ATG GTA GTA GC – 3'

LN280
5' – GAT TAC TCT GAT GTC TGT CCT AGT ACG AGT CAG TCT CTC CA – 3'

NC112
5' – TCT ACA TGC CGT ACA TAC GGA ACG TAC GGA GCG ATT CGA CTG ACA GTC GTA ACC TCA – 3'

D336
5' – (N6)G(N6) CT(N6) C(N6)G (N6)C(N6) GCG ACA ACT GTA CCA TCT GTG TCC ATG GT – 3'

D405
5' – (C23)(C23)(C23) (C23)(C23)(C23) (C23)(C23)(C23) (C23)AT CTG TGT CCA TGG T – 3'

D429
5' – (N6)G(N6) CT(N6) C(N6)G (N6)C(N6) (C131)AT CTG TGT CCA TGG TAG TAG C – 3'

FIG._19B

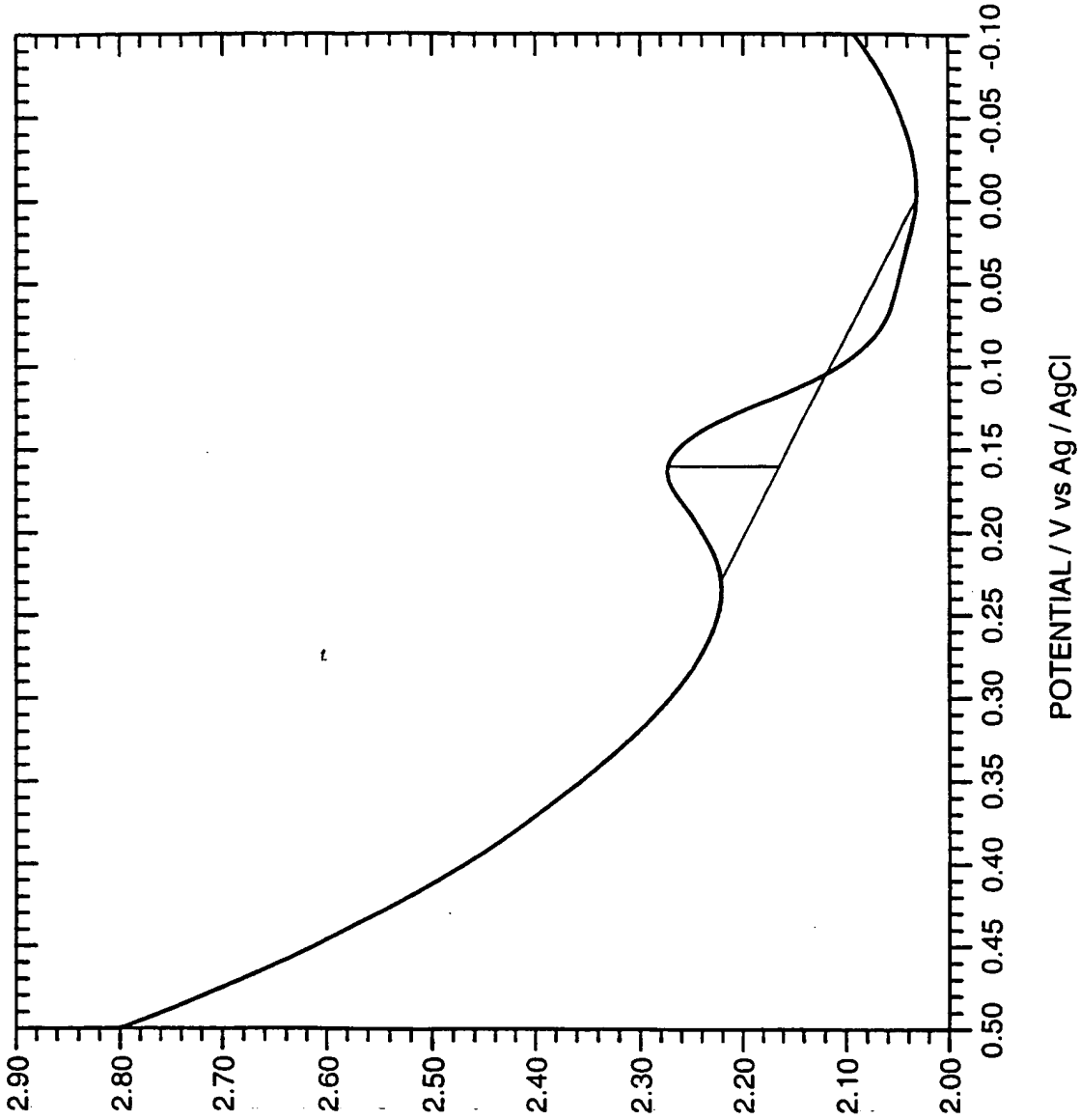


FIG._20A

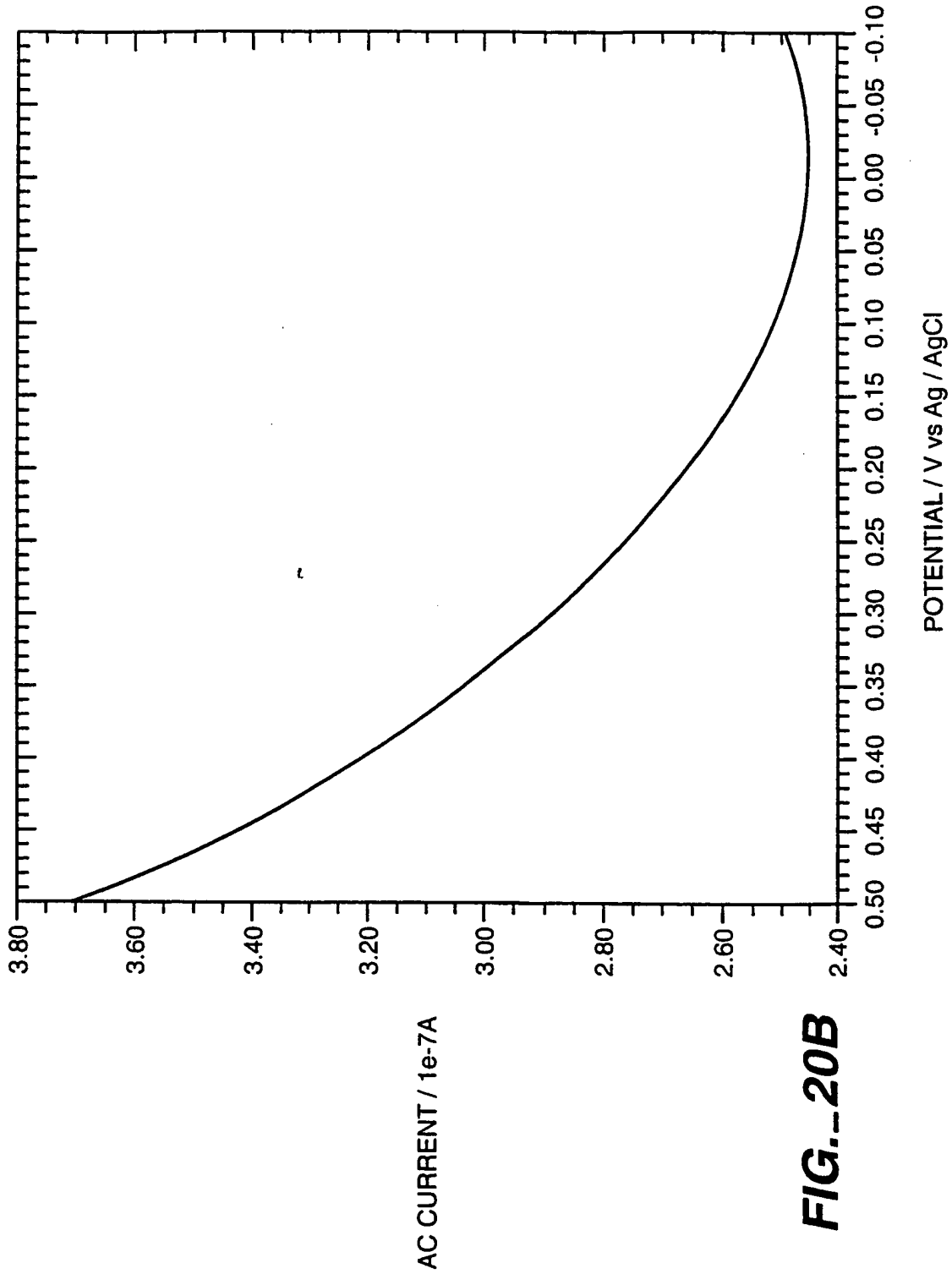


FIG._20B

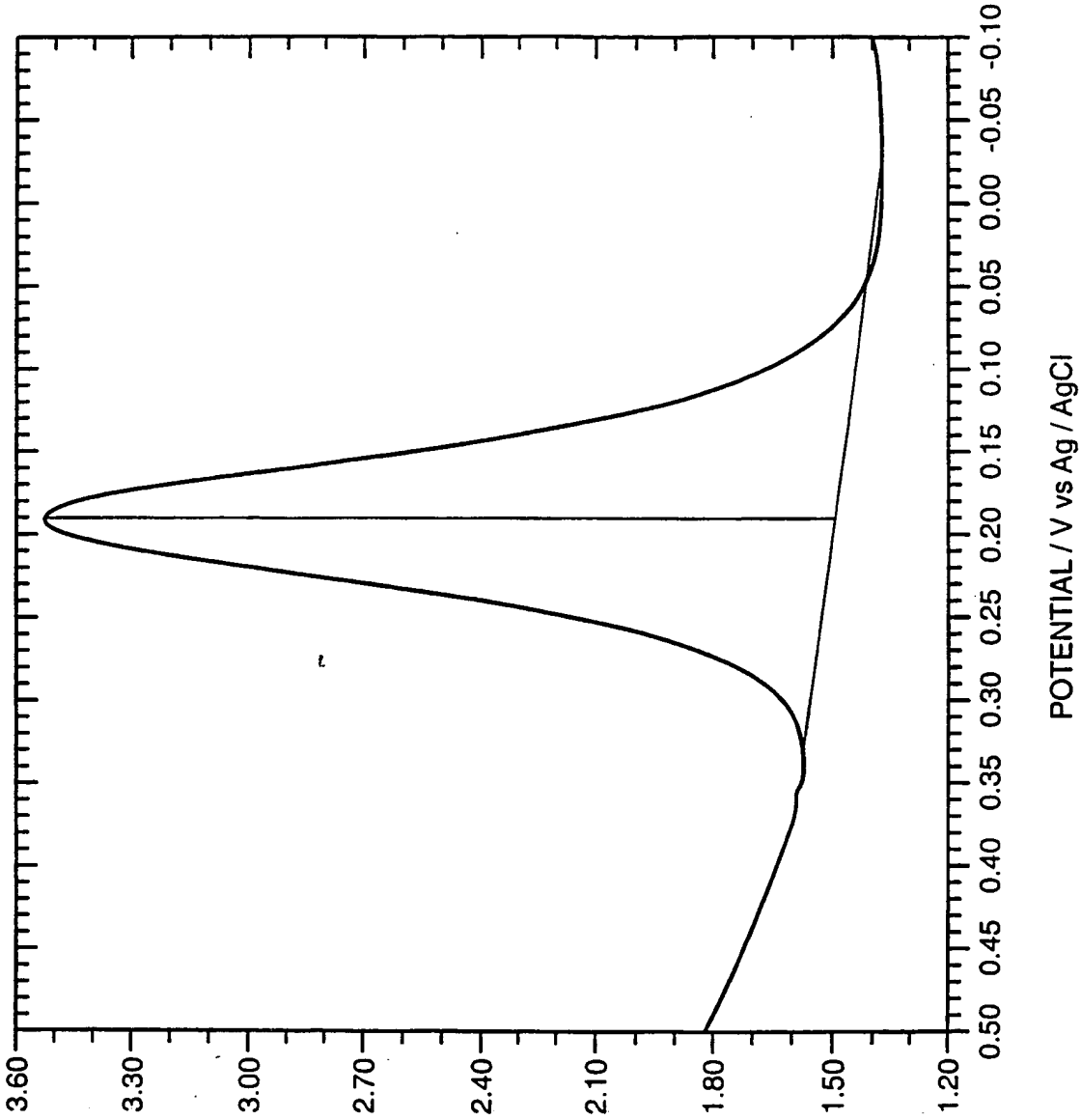


FIG._20C

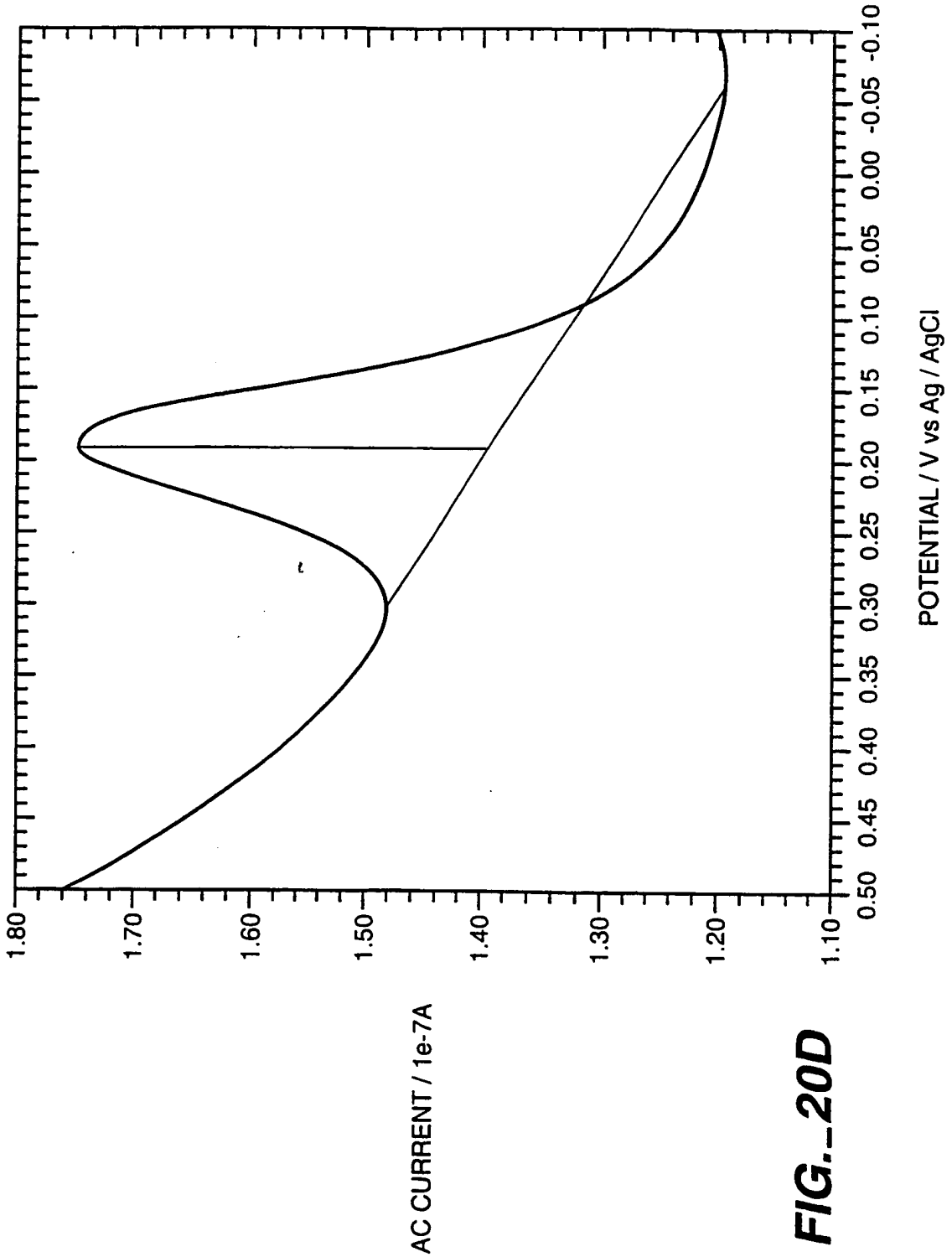


FIG._20D

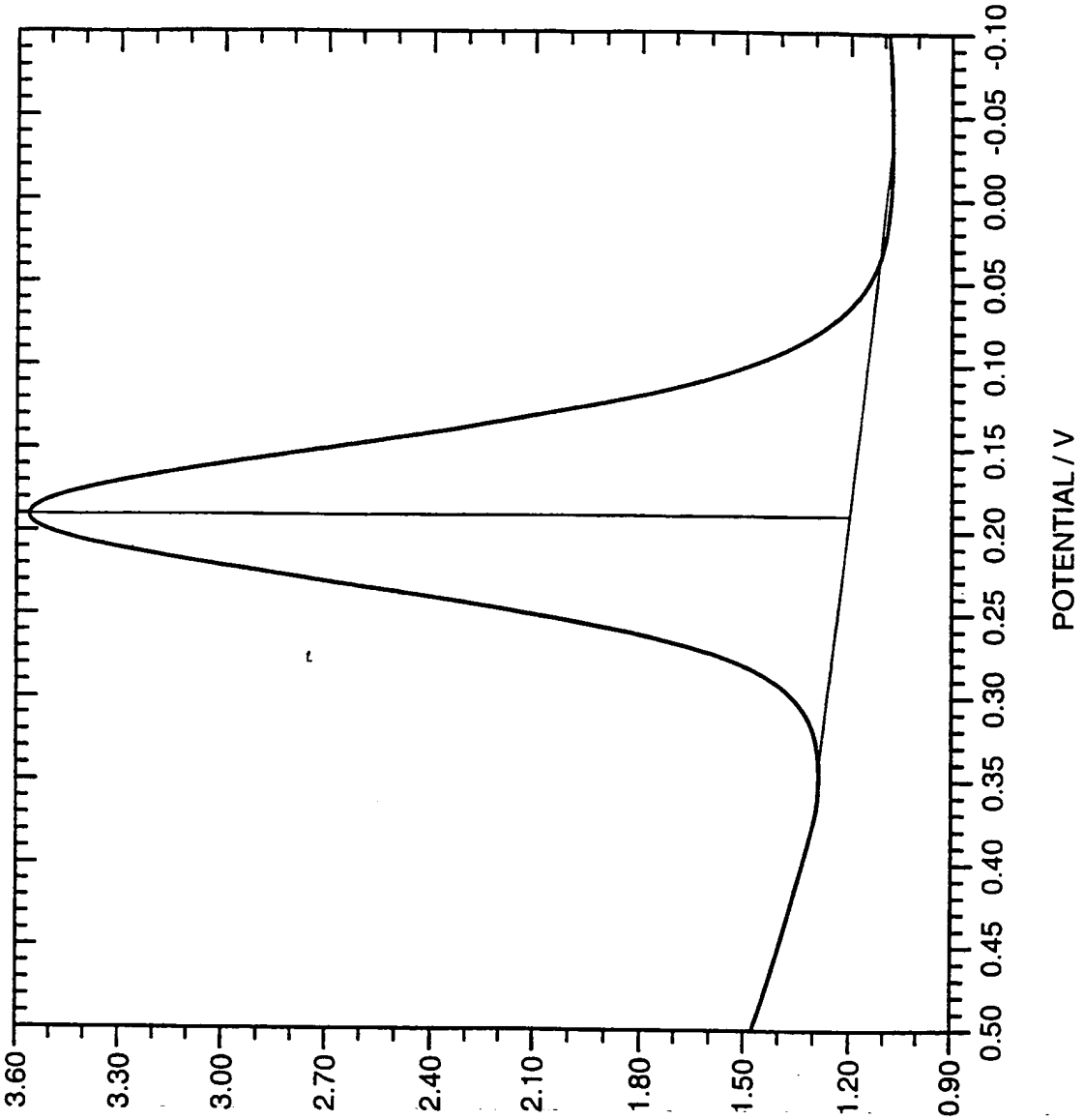


FIG._20E

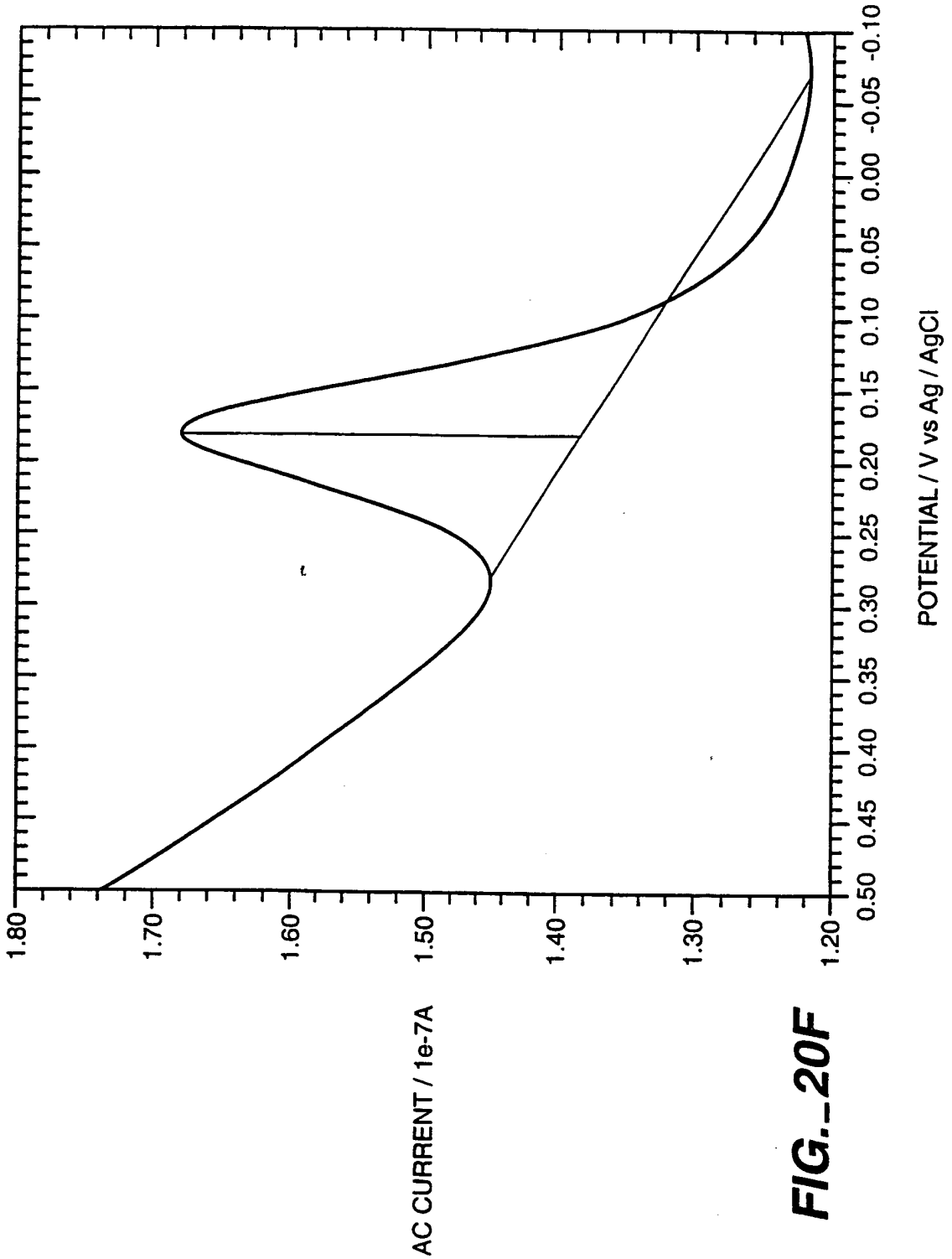


FIG._20F

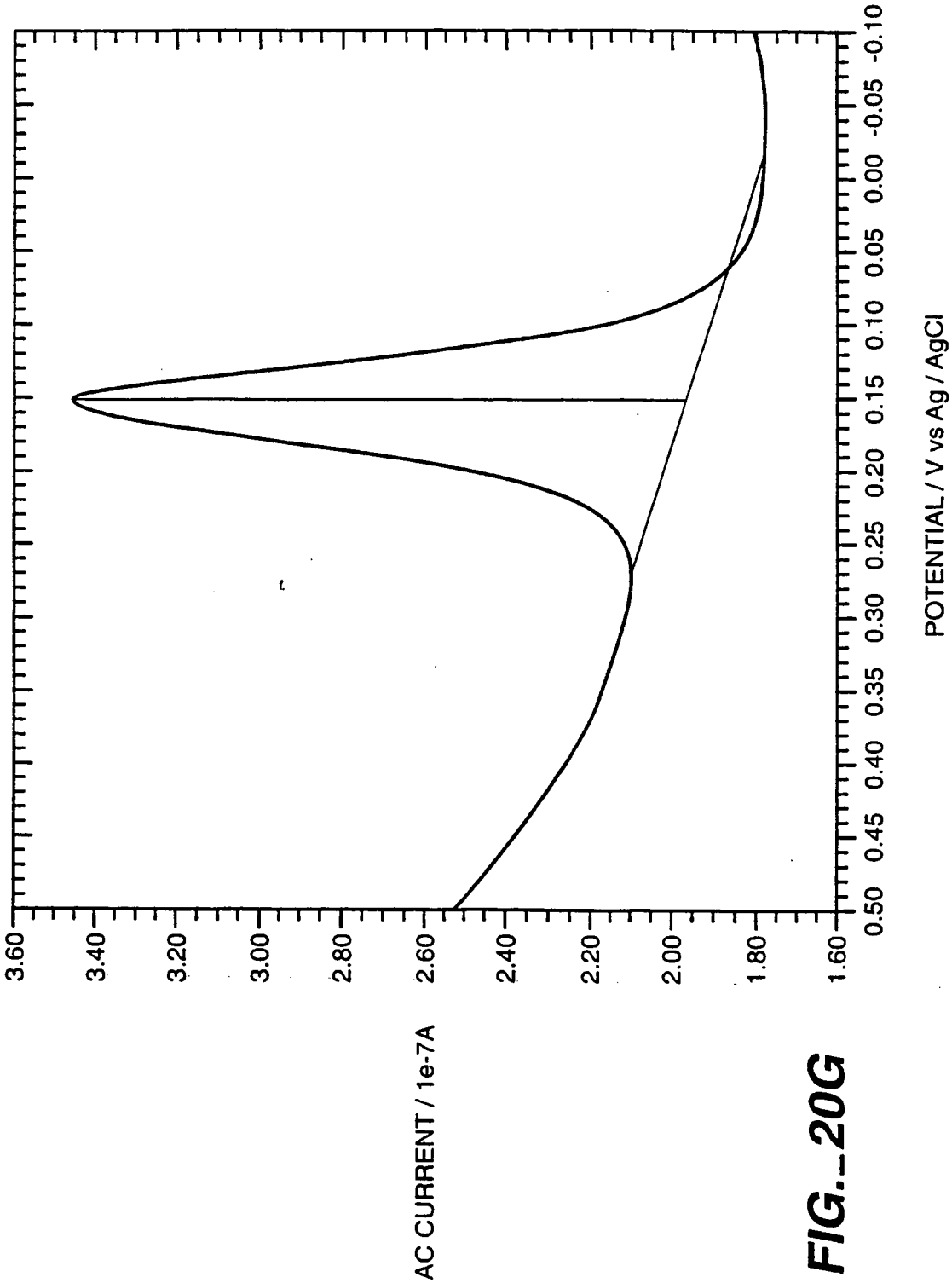


FIG._20G

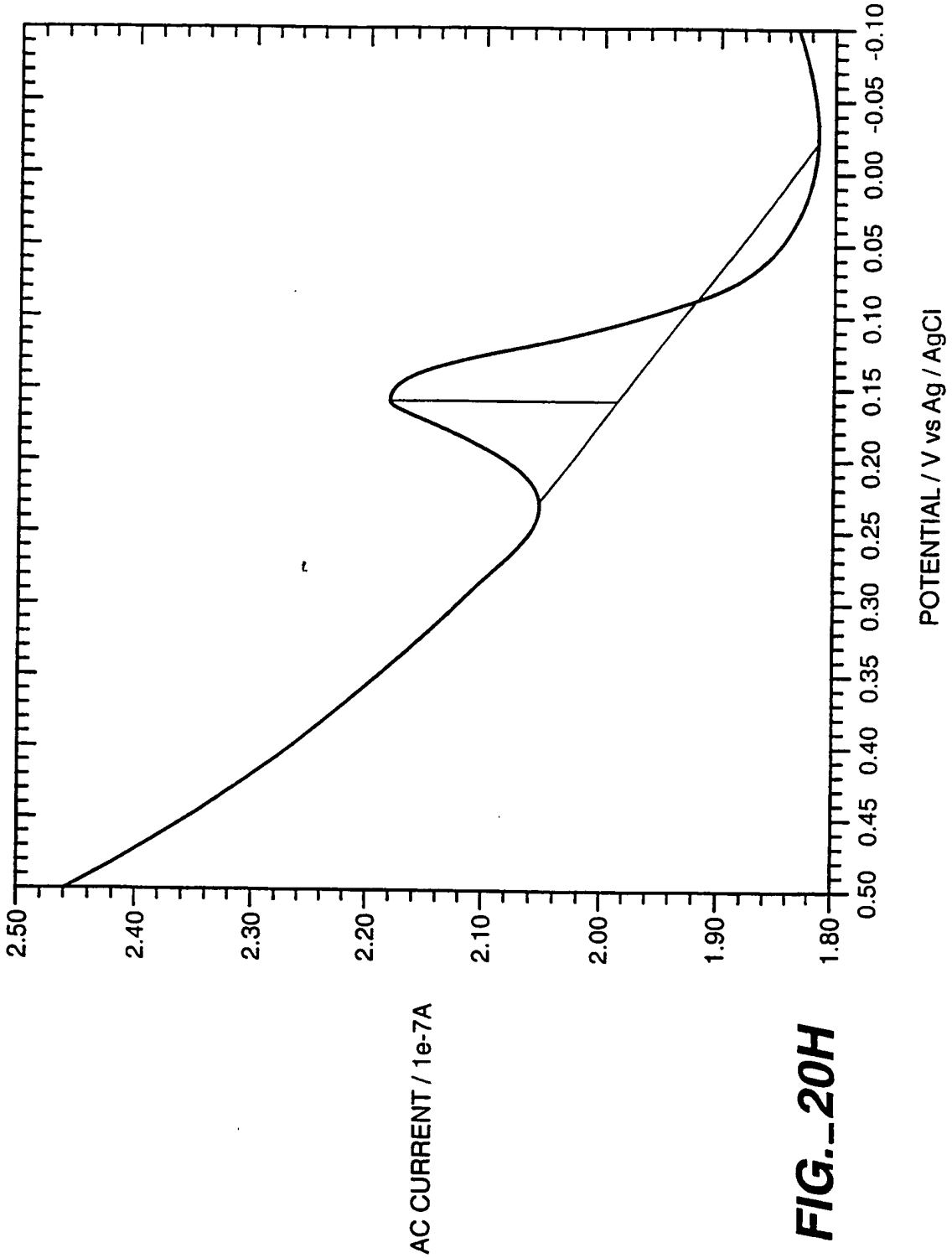


FIG._20H

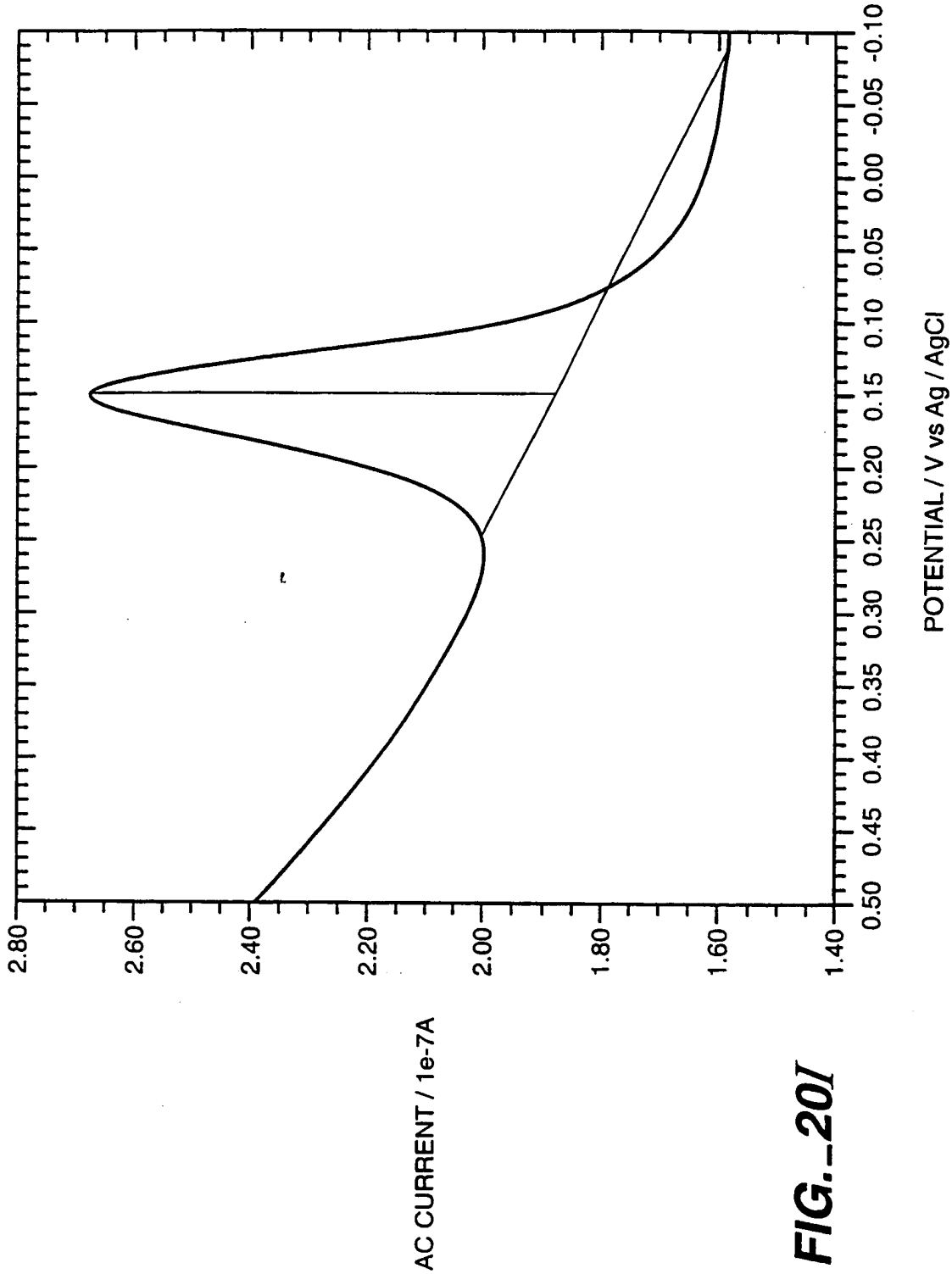


FIG._20I

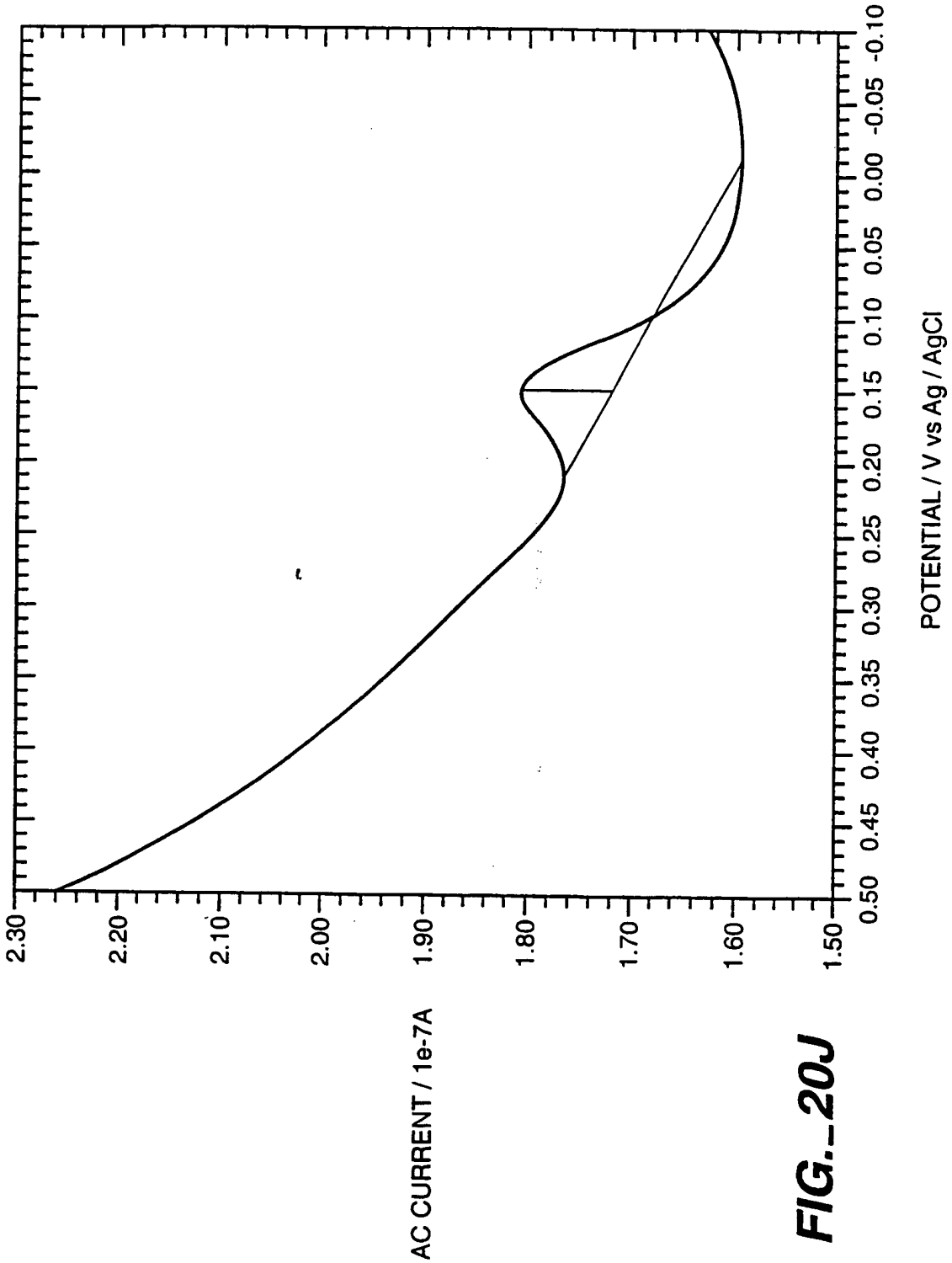


FIG._20J

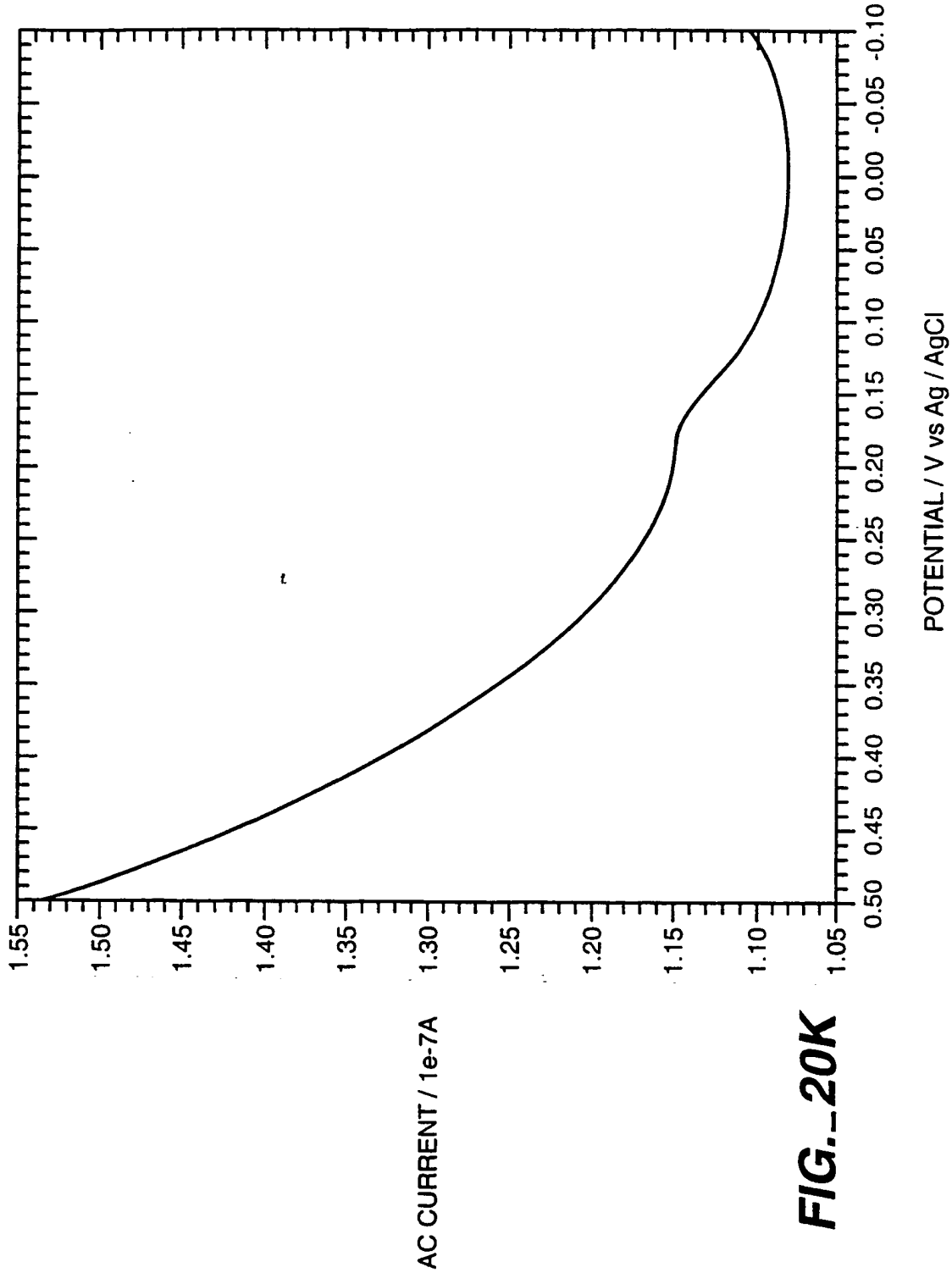


FIG._20K

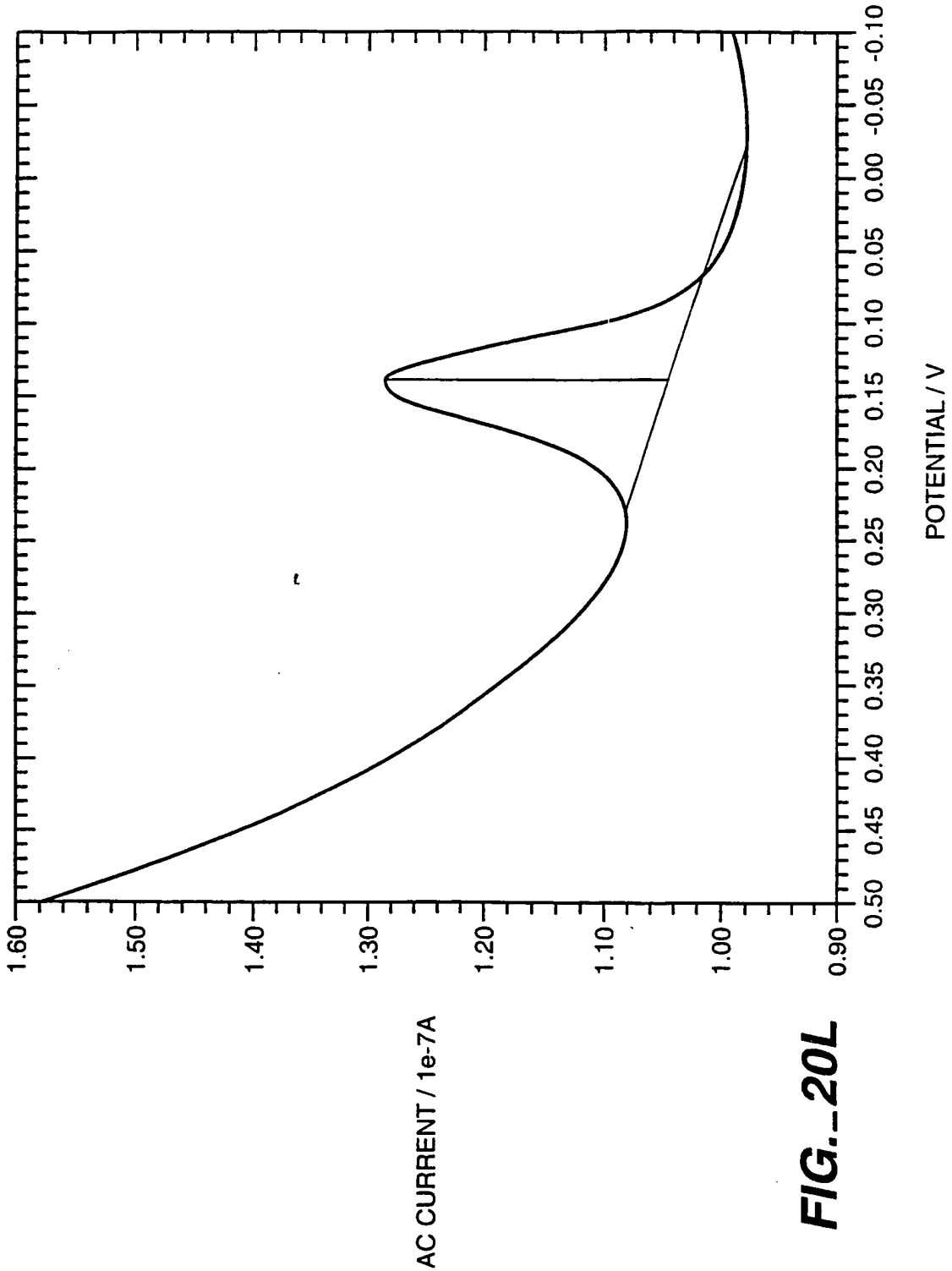


FIG._20L

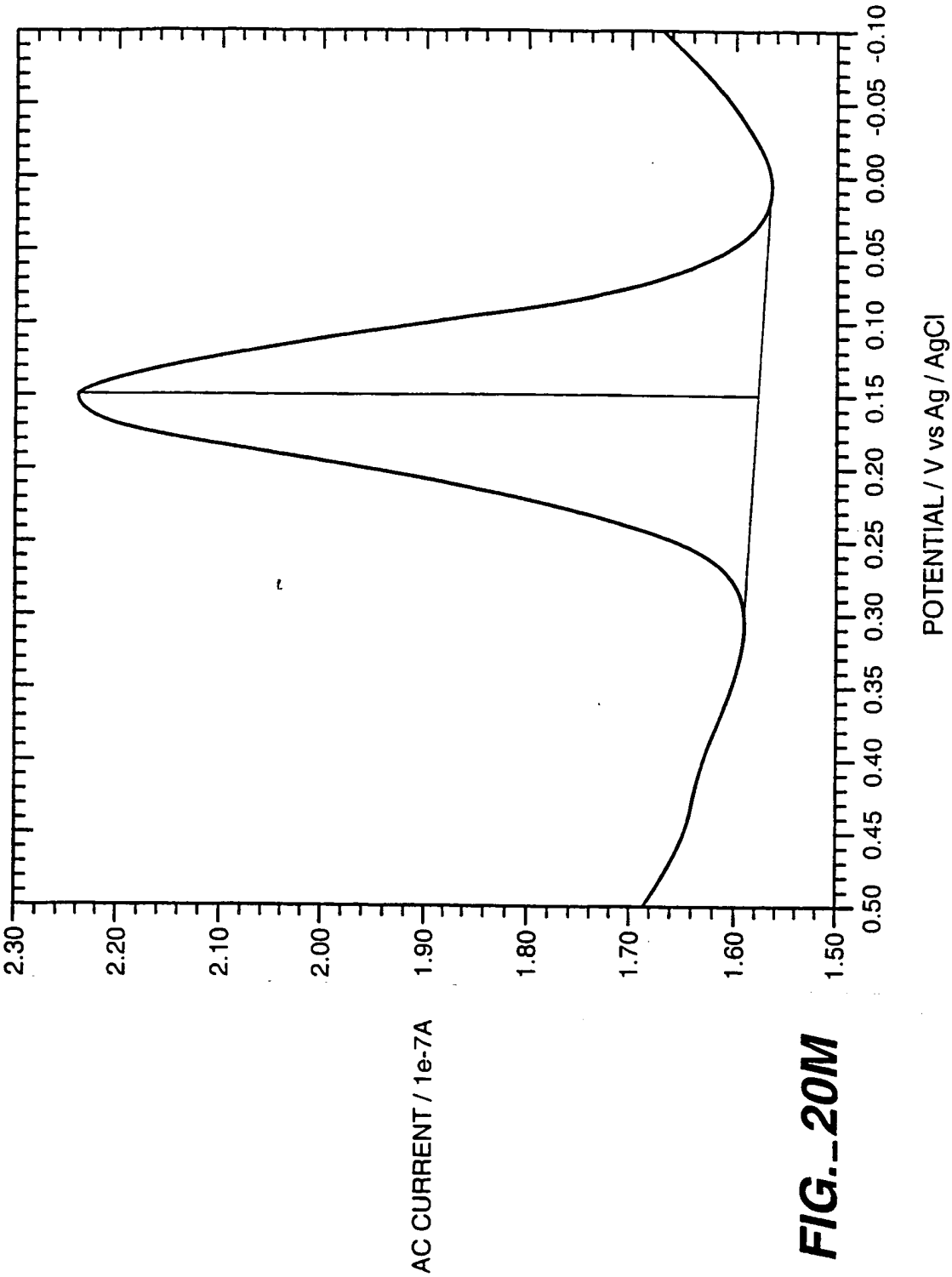


FIG._20M

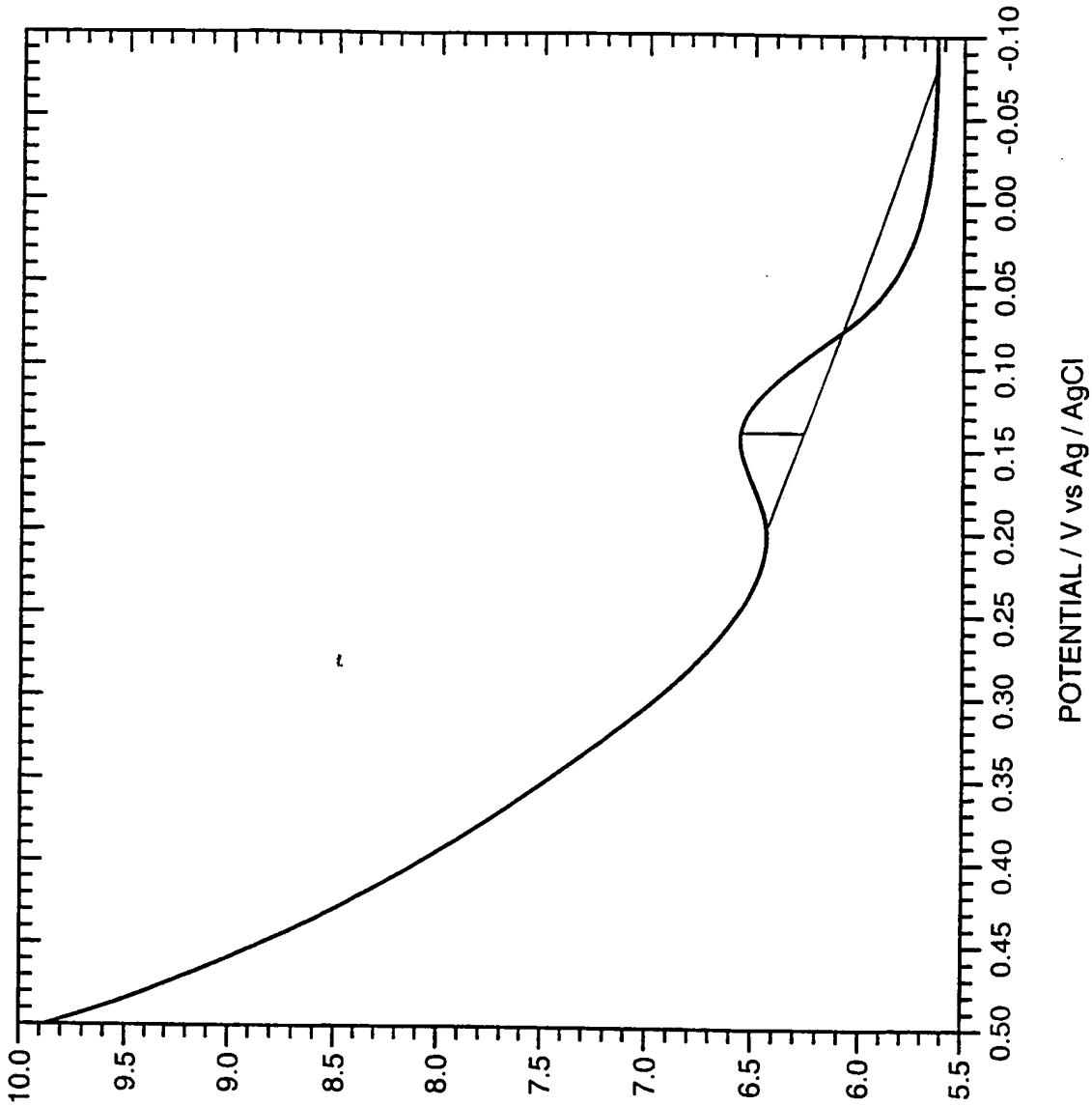


FIG._20N

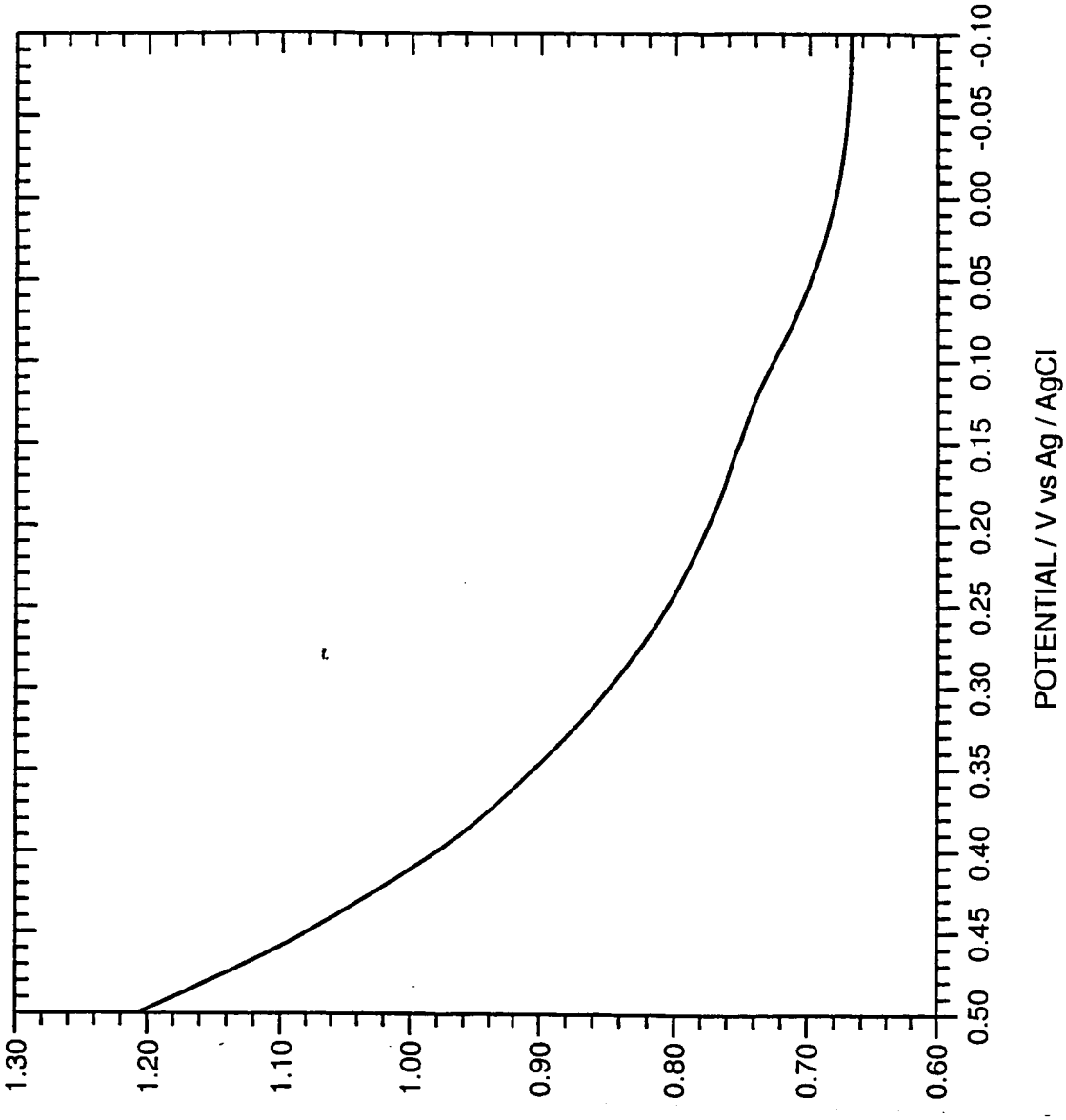


FIG._20

D456
5' – (N6)G(N6) CT(N6) C(N6)G (N6)C(N6) TTC TGC ACC GTA GCC ATG AAA GAT TGT ACT GAG – 3'

D368
5' – (H2)CC TTC CTT TCC ACA U – 4 UNIT WIRE (C11) – 3'

HIVCOMP
5' - ATG TGG AAA GGA AGG ACA CCA AAT GAA AGA TTG TAC TGA GAG ACA GGC TAA TTT TTT AGG GAA GAT CTG G – 3'

HIVLIG
5' – CCA GAT CTT CCC TAA AAA ATT AGC CTG TCT CTC AGT ACA ATC TTT CAT TTG GTG T – 3'

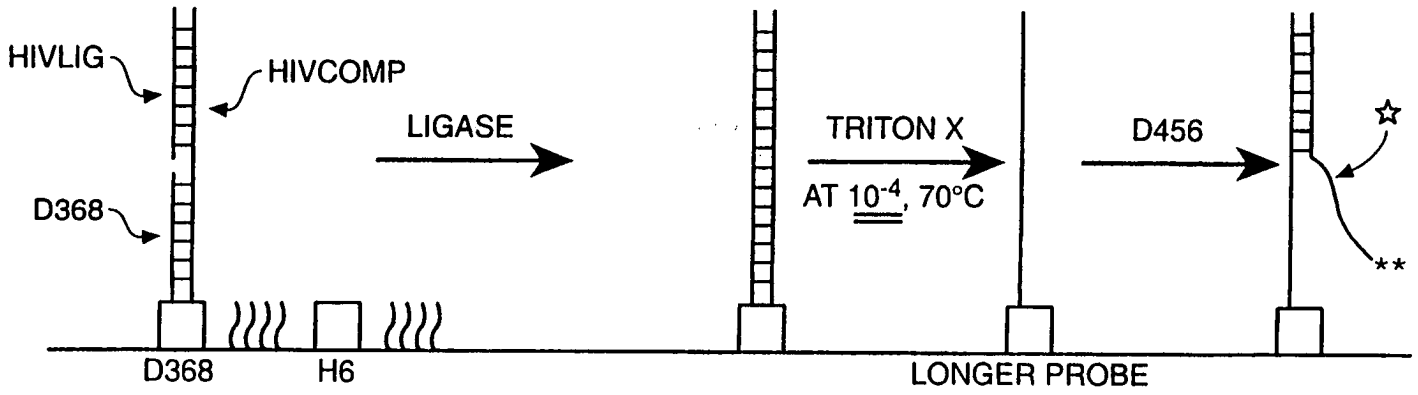


FIG._21

MEASURER	FILE	ELECTRODE #	HYBRID CODE	ip (nA)	AVERAGE ip (nA)	STDEV ip (nA)	POTENTIAL (mV)	ip (nA)	POTENTIAL (mV)
A	1	7	1-	0			–	1.593	70
B	3	8	EU2+reg	0			–		
B	4	6	helpers+reg	0	0.36	0.71	–		
JB	3	5	system	1.42			60		
A	2	3	1+	0.7449			160		
B	1	4	rRNA EU2+reg	0.196			140		
JB	1	1	helpers+reg	0.8547	0.63	0.29	160		
JB	2	2	system	0.722			160		
A	5	13	2-	0.3146			160	0.2506	70
A	6	15	EU2+EU1, 2 reg	0.3441			170	0.8442	80
JB	4	14	helpers+reg	0	0.19	0.17	–		
JB	6	16	system	0.11			160		
A	3	11	2+ rRNA EU2+	0.586			170	0.05	70
A	4	12	EU1, 2 reg helpers	1	1.06	0.51	160		
B	2	9	+ reg system	1.6			150	2.4	50
A	8	22	3-	2.661			160		
B	5	23	(2) 20-Fc	0.9	3.03	2.99	160	2.8	120
B	8	24	ETMs+reg	1.2			160		
JB	7	21	system	7.376			150		
A	7	18	3+	1.756			170	0.4778	350
B	6	19	rRNA+ (2) 20-Fc	0.77	2.99	2.76	120		
B	7	20	ETMs+reg	7			150		
JB	5	17	system	2.448			160		
A	11	29	4-	1.426			180	0.1	70
B	10	32	(2) 40-Fc	3	2.42	1.11	150		
B	11	31	ETMs+reg	3.7			150		
JB	9	30	system	1.571			170		
A	9	25	4+	12.49			160		
A	10	26	rRNA+ (2) 40-Fc	9.278	7.46	4.16	160		
B	9	28	ETMs+reg	4			130		
JB	8	27	system	4.088			150		

FIG._22A

MEASURER	FILE	ELECTRODE	HYBRID CODE	$2/\pi * i_p$ (nA)			E_0 (mV)	$2/\pi * i_p$ (nA)	E_0 (mV)
				RAW DATA	AVERAGE	STDEV			
JZ A	2 3	46 47	5-	1.041 2.811	1.93	1.25	170 170	4.465	60
A JZ A	1 1 2	41 43 44	5+	5.7 1.862 2.613	3.39	2.03	170 170 180	0.96	60
A JZ A	5 5 6	53 55 56	6-	0.6566 0.8548 5.167	2.23	2.55	170 170 180	2.1 1.64	60 60
JZ A JZ	3 4 4	49 50 52	6+	5.799 8.468 3.187	5.82	2.64	170 180 180		
JZ A JZ	7 8 8	61 62 64	7-	0.1988 1.382 0.6104	0.73	0.60	160 170 160	1.147 1.04 0.1958	60 50 60
JZ A	6 7	58 59	7+	1.459 1.042	1.25	0.29	160 160	2.38	60
JZ A	10 11	70 71	8-	0.3208 0.7994	0.56	0.34	160 190	0.504 2.22	60 60
A JZ A	9 9 10	65 67 68	8+	3.297 1.492 2.841	2.54	0.94	170 160 170	0.71	60
JZ	12	76	9-	1.215	1.22	#DIV / 0I	170	4.414	50
JZ A	11 12	73 74	9+	3.768 5.592	4.68	1.29	170 170	0.7741 0.53	50 60
JZ A	14 14	78 80	10-	2.842 7.4	5.12	3.22	170 170	2.319	50
A JZ	13 13	77 79	10+	5.582 4.337	4.96	0.88	170 160	3.173	50

FIG._22B

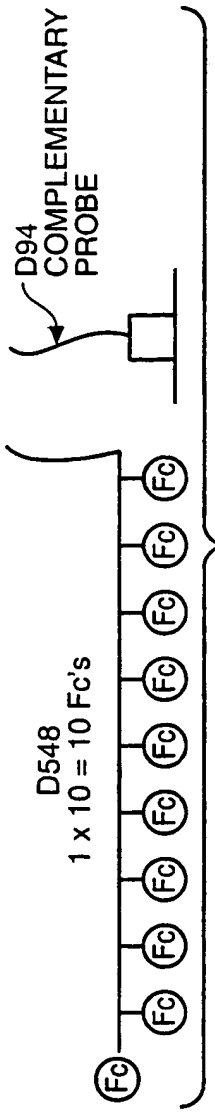


FIG._23C

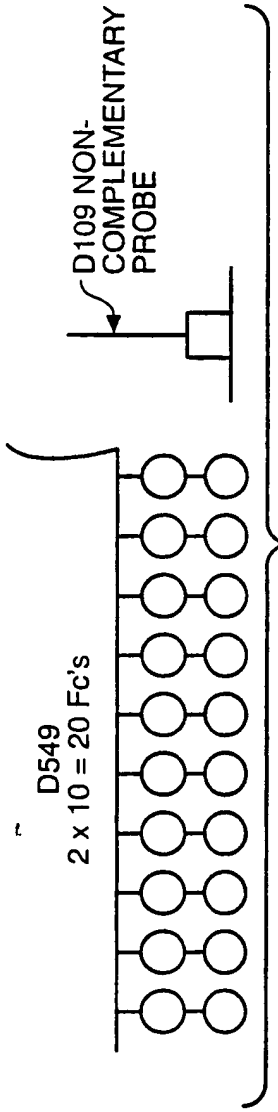


FIG._23D

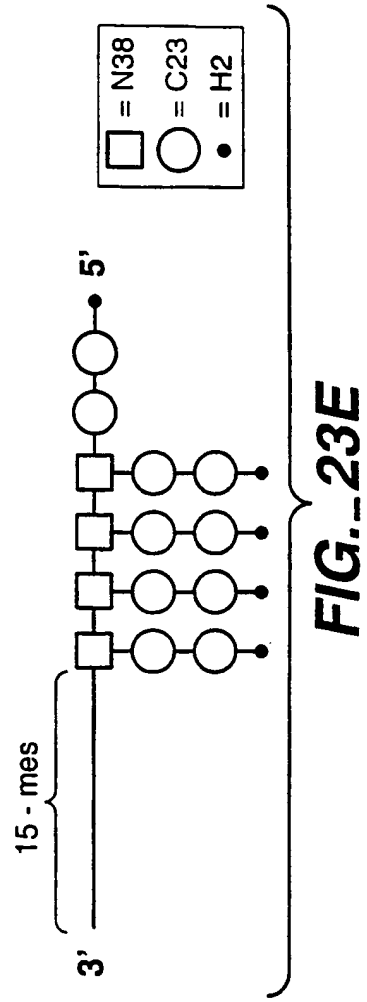


FIG._23E

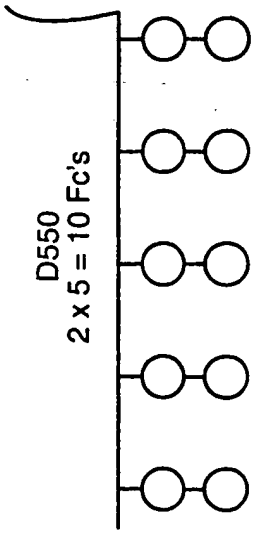


FIG._23A

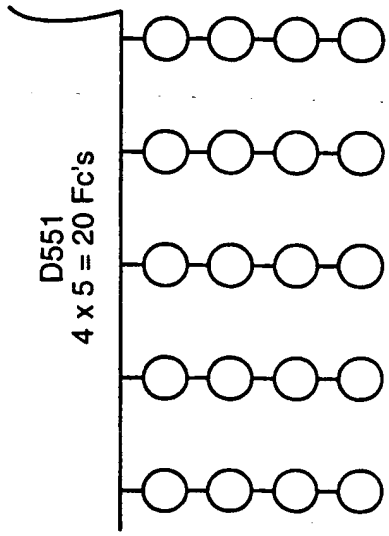


FIG._23B

MEASURER	EXPT	FILE	ELECTRODE	SURFACE	HYBRID	$2/\pi * i_p$ (nA)	E_0 (mV)	AVERAGE $2/\pi * i_p$ (nA)	STDEV $2/\pi * i_p$ (nA)
A	409	1	1	"+" Surface 2:2:1 D94 / H6 / M44*, total thiol = 833 uM	D548 (1x10)**	22.6	150	14.5	5.8
A	409	17	17			9.622	200		
Z	73	8	8			14.51	100		
Z	73	22	24			11.15	110		
A	409	8	7		D549 (2x10)	53.52	200	60.6	12.9
A	409	22	23			71.13	220		
Z	73	1	2			71.66	110		
Z	73	17	18			45.9	120		
A	409	4	3		D550 (2x5)	72.4	190	45.5	18.9
A	409	18	19			30.67	210		
Z	73	7	6			44.49	120		
Z	73	19	22			34.43	120		
A	409	7	5		D551 (4x5)	105.8	210	74.9	23.5
A	409	19	21			48.66	230		
Z	73	4	4			70.42	130		
Z	73	18	20			74.77	130		
A	409	9	9	"-" Surface 2:2:1 D109 / H6 M44*, total thiol = 833 uM	D548 (1x10)	5.665	200	1.6	2.7
A	409	25	25			0.6443	250		
Z	73	16	16			0.0864	120		
Z	73	30	32			0	–		
A	409	16	15		D549 (2x10)	10.24	230	8.3	5.9
A	409	30	31			14.57	260		
Z	73	9	10			7.881	130		
Z	73	25	26			0.5476	140		
A	409	12	11		D550 (2x5)	4.513	230	3.7	1.6
A	409	26	27			4.264	260		
Z	73	15	14			4.553	150		
Z	73	27	30			1.314	140		
A	409	15	13		D551 (4x5)	10.31	240	9.0	6.9
A	409	27	29			17.46	280		
Z	73	12	12			7.445	160		
Z	73	26	28			0.8812	90		

* Note: M44 = M43.

** Also note: (n x m) means there are m bristles, each with n Fc's.

FIG._23F

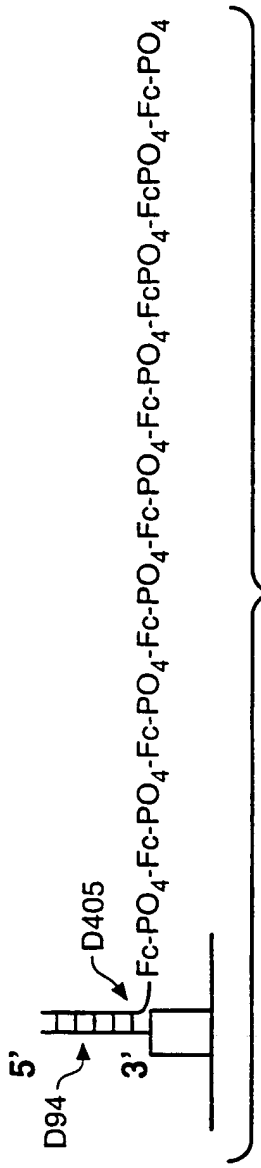


FIG._24A

MEASURER	EXPT	FILE	ELEC-TRODE	SURFACE	HYBRID	$2/\pi * i_p$ (nA)	E_o (mV)	AVERAGE $2/\pi * i_p$ (nA)	STDEV $2/\pi * i_p$ (nA)
A	52	1	1	"+" Surface 2:2:1 D94 / H6 / M44*; total thiol = 833 uM	10 uM D405 in 6x SSC w/50% FCS	4.81	170	18.04	14.53
A	52	4	3			20.63	180		
Z	384	1	2			37.42	170		
Z	384	4	4			9.31	160		
A	52	7	5	"-" Surface 2:2:1 D109 / H6 / M44*, total thiol = 833 uM	10 uM D405 in 6x SSC w/50% FCS	0.1	160	3.12	4.70
A	52	10	7			9.97	160		
Z	384	5	6			0	–		
Z	384	8	8			2.425	180		

*NOTE: M44 = M43

FIG._24B

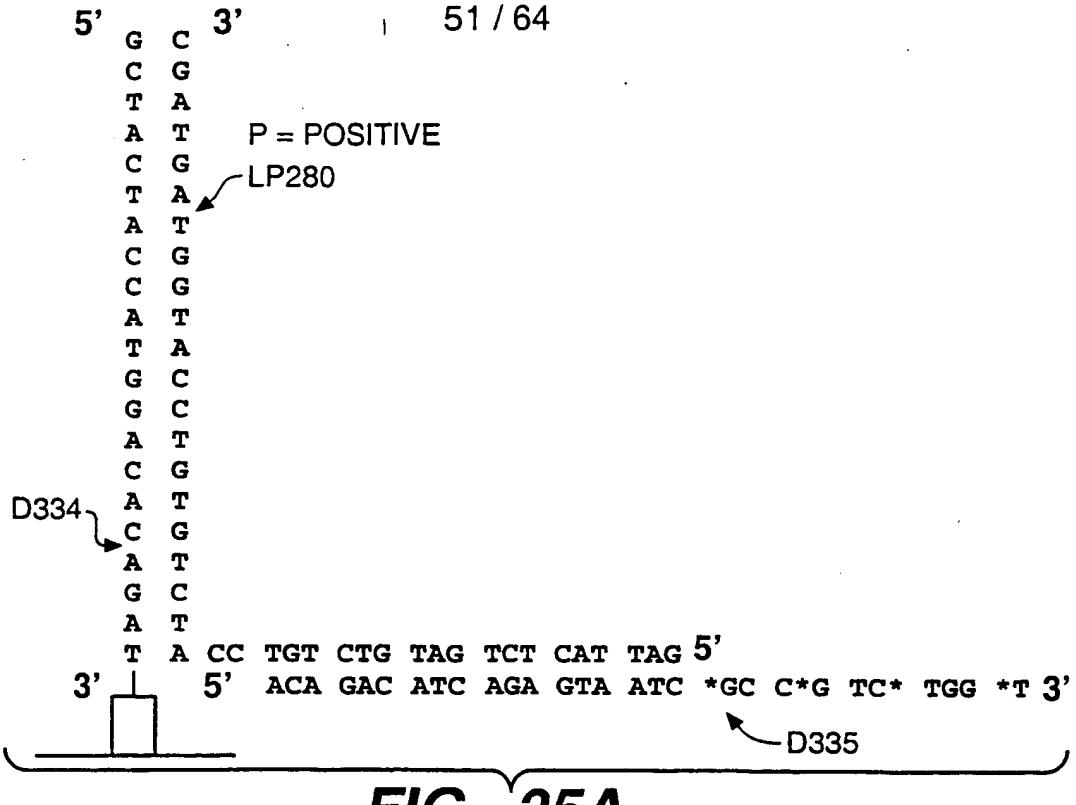


FIG._25A

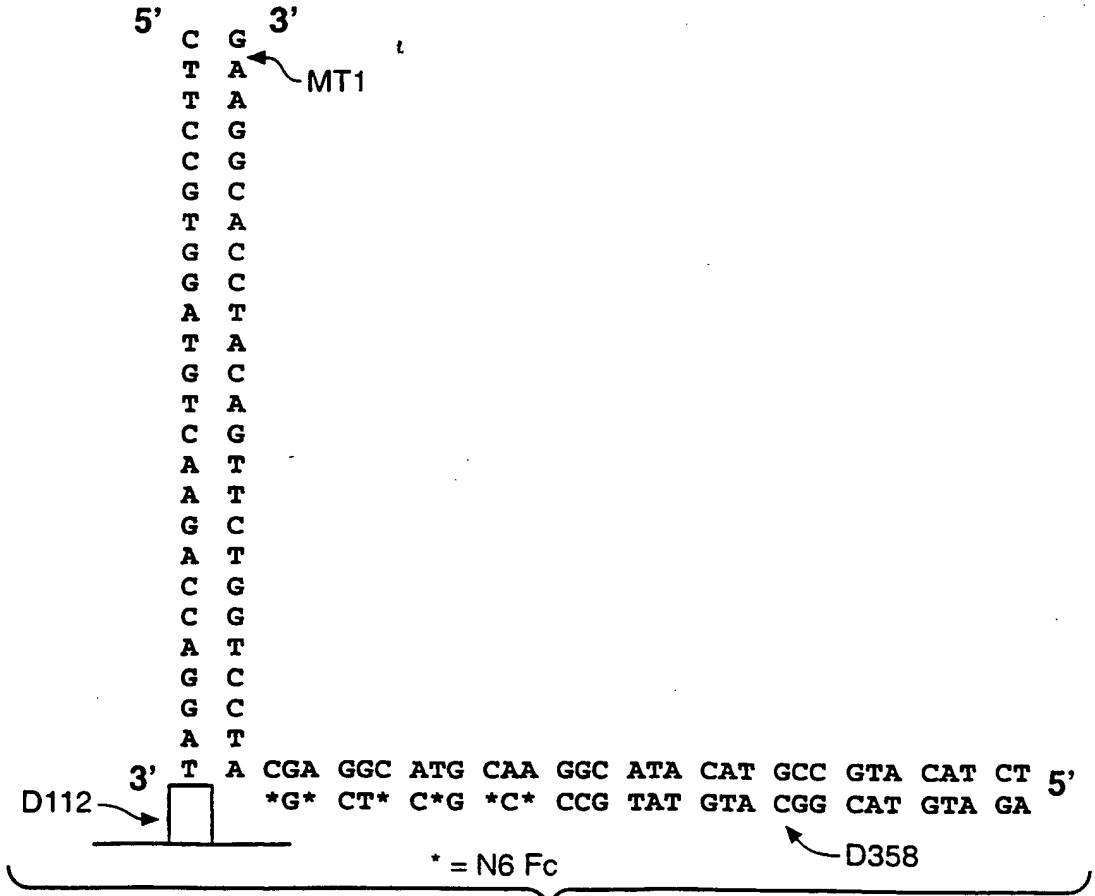


FIG._25B